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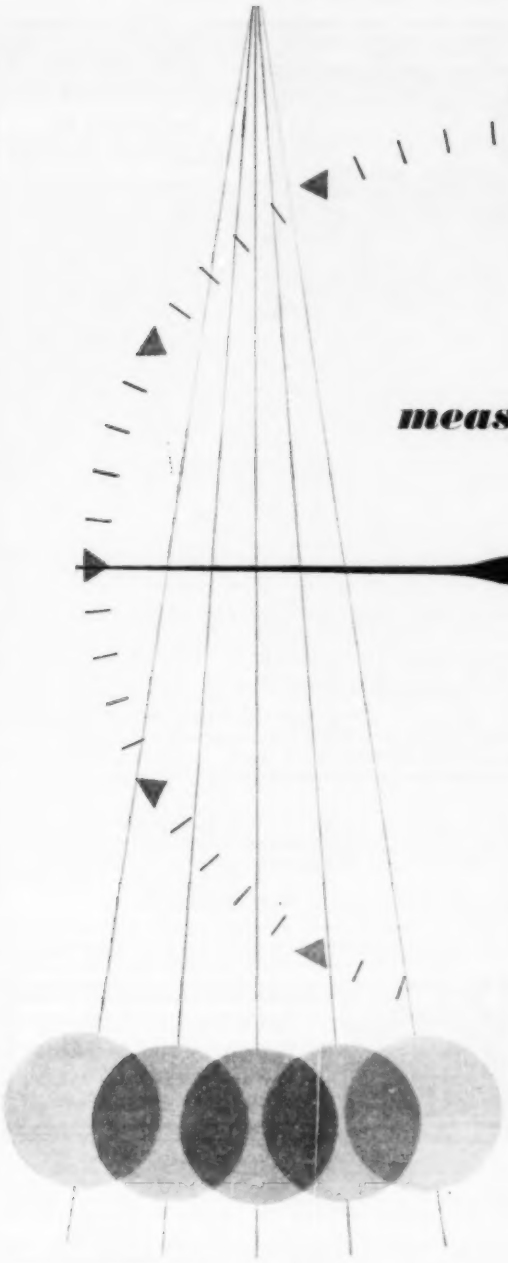

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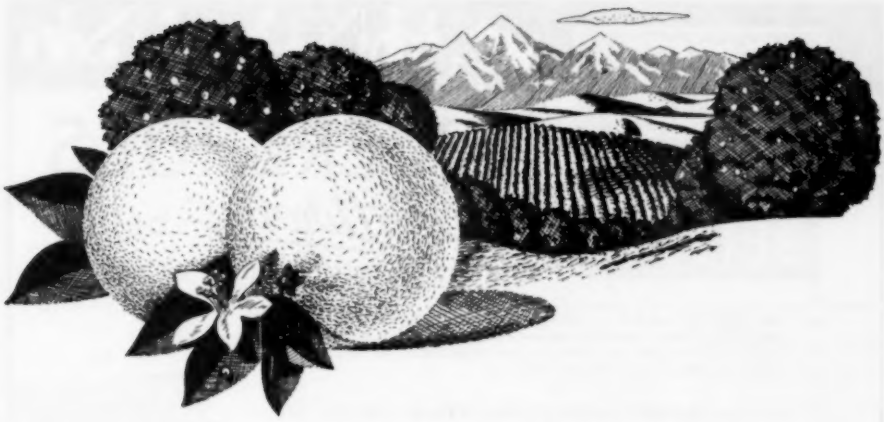

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

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Aureotracin: A Combination of Aureomycin and Bacitracin*

By HENRY WELCH, W. A. RANDALL, and C. W. PRICE

An insoluble compound of bacitracin and aureomycin designated aureotracin is described. The antibacterial properties of both antibiotics are preserved. In this combination the bacitracin is much less nephrotoxic than the soluble drug. This combination may be safely injected into man and produces prolonged blood and urine concentrations.

AUREOMYCIN has established itself as one of the most versatile antibiotics available. Although it is efficacious by all routes of administration, intramuscular injections are painful and intravenous injections entail hazards that limit its use. Oral administration is widely used and has proved highly effective; however, a preparation that could be injected intramuscularly with a minimum of pain would be of considerable value. Bacitracin also produces pain at the site of injection and has a further disadvantage because of its nephrotoxicity. During some experimental work on the synergistic action of antibacterial agents (1, 2), it was noted that combinations of dilute solutions of the two antibiotics remained clear, but when more concentrated solutions were mixed in aqueous solution, a dense golden yellow precipitate formed rapidly. Collection of this precipitate and injection into experimentally infected animals showed that the antibacterial activity of both antibiotics was retained. With the possibility that a new compound had been formed by the mixture of these two drugs under the above conditions, a small lot of the material was prepared for further study.

EXPERIMENTAL

A total of 3.75 Gm. of bacitracin (166,000 units) dissolved in 37.5 ml. of water was mixed with 0.2 Gm. aureomycin previously dissolved in 20 ml. of water. The precipitate was collected, washed with water, frozen, and dried. A golden yellow powder resulted which was insoluble in water and ordinary organic solvents but quite soluble in dilute alkalies and acids. Suspensions of this material in oil showed no aureomycin crystals of the type readily demonstrated when a physical mixture of aureomycin and bacitracin was examined. The powder was placed on a filter and washed repeatedly with water until the amount going into solution (0.02%) appeared to be constant. This figure is considerably below the 1% solubility of aureomycin and 50% solubility of bacitracin. After freeze-drying the resulting powder was light yellow, amorphous, and highly insoluble in water. The infrared absorption spectrum revealed many points in common with aureomycin and bacitracin, yet definite differences were apparent. The term

* Received July 21, 1950, from the Food and Drug Administration, Federal Security Agency, Washington, D. C.

"aureotracin" was coined as descriptive of the new preparation. A large batch was then prepared and tests conducted in an attempt to evaluate it. It is of interest that some batches of bacitracin do not react with aureomycin to form aureotracin. A study of several batches of bacitracin indicated that the fraction of bacitracin reacting with aureomycin is acid-precipitable. This fraction, present in certain lots of bacitracin, is precipitated at pH 5.0, has about 50% of the potency of the parent substance, and is in itself nephrotoxic.

The toxicity of bacitracin in white mice is fairly high and since aureotracin contains relatively large amounts of bacitracin, toxicity tests were conducted. Assay of the aureotracin showed 300 μg./mg. activity as aureomycin and 25 u./mg. as bacitracin. A physical mixture of aureomycin and bacitracin containing these proportions was prepared for use as a control. Both the aureotracin and the physical mixture were finely ground and suspended in sterile sesame oil. Groups of 10 white mice weighing 20 Gm. each were given from 5 to 40 mg. of the two preparations intraperitoneally and observed for ninety-six hours. Previous experience showed that from 5 to 6 mg. of the bacitracin used in preparing these substances was invariably lethal to 20-Gm. white mice on intraperitoneal injection. The results are given in Table I.

TABLE I.—COMPARATIVE TOXICITY OF AUREOTRACIN AND A PHYSICAL MIXTURE OF AUREOMYCIN PLUS BACITRACIN FOLLOWING INTRAPERITONEAL INJECTIONS OF WHITE MICE

Mg. per Mouse Injected	Physical Mixture of Aureomycin and Bacitracin		Aureotracin	
	No. Mice	No. Dead	No. Mice	No. Dead
5	10	2	10	0
10	10	10	10	0
20	10	10	10	0
40	10	10	10	0

The toxicity of the physical mixture (Table I) was what one would expect from its bacitracin content, while more than four times as much bacitracin in aureotracin failed to kill any of the injected mice.

The effect of aureotracin and the physical mixture of aureomycin and bacitracin on the kidneys was determined. White mice weighing 20 Gm. were injected in groups of five with aureotracin or the physical mixture, both suspended in oil, and urines collected at thirty minutes, two, four, six, twenty-four, twenty-eight, and thirty hours to determine the amount of aureomycin excreted. All mice remaining alive were sacrificed, the kidneys removed and studied grossly and microscopically for pathological change. The results appear in Table II.

During the first two hours the excretion of aureomycin following injection of the physical mixture of aureomycin and bacitracin (Table II) did not materially differ from that excreted following injection of aureotracin. The excretion at twenty-four hours, however, as well as total excretion following injection of the 5-mg. dose shows that aureotracin releases more aureomycin than the physical mixture. In order to determine whether aureotracin has a more prolonged activity in the body than the physical mixture of aureomycin and bacitracin, white mice were injected with aureomycin, aureotracin, and a physical mixture of aureomycin-bacitracin, all suspended in sesame oil. Challenging doses of *Salmonella typhosa* were given two and one-half, five, and twenty-four hours following administration of the drugs. The results are given in Table III.

It is apparent that when experimental infections of *S. typhosa* in white mice are induced two and one-half to twenty-four hours after the injection of aureomycin, aureotracin, or a physical mixture of aureomycin and bacitracin, aureotracin is able to protect greater numbers of mice for longer periods of time than the other two preparations. When in-

TABLE II.—EXCRETION OF AUREOMYCIN FOLLOWING INTRAPERITONEAL INJECTION OF AUREOTRACIN AND A PHYSICAL MIXTURE OF AUREOMYCIN AND BACITRACIN

Drug	Dose, Mg.	No. of Mice	Dead, %	Renal Damage	Micrograms Aureomycin Excreted/Mouse—Hours						
					½	2	4	6	24	28	30
Physical mixture of aureomycin and bacitracin	20	5	100	...	...	..	..	..	..	..	..
	10	5	100	...	..	..	..	..	..	..	..
	5	5	40	Severe	2.8	0	0	4	42	1.2	8.7
Aureotracin	20	5	0	0	2.8	8	6	1	68	34	30
	10	5	0	0	2.8	6	5	9	70	3	..
	5	5	0	0	0	3	7	8	65	1	17

TABLE III.—PROTECTION BY AUREOMYCIN, AUREOTRACIN, OR A PHYSICAL MIXTURE OF AUREOMYCIN-BACITRACIN OF WHITE MICE INFECTED WITH *Salmonella typhosa*

Drug Administered	Dose, Mg.	No. of Mice	Time in Hours between Administration of Drug and Infection of Mice	Survival, %
Aureotracin	2	50	2.5	80
	2	50	5	90
	2	50	24	90
Aureomycin	2	50	2.5	80
	2	50	5	65
	2	50	24	52
Aureomycin-bacitracin mixture	2	50	2.5	80
	2	50	5	65
	2	50	24	32

fection was induced five to twenty-four hours after aureotracin was injected, 90% of the mice survived, while only 32% to 65% of the mice survived when mice treated with aureomycin alone or a physical mixture of aureomycin and bacitracin were similarly infected.

In view of the fact that both aureomycin and bacitracin had been used repeatedly in humans, it was decided to observe the effects of aureotracin on three volunteers. Accordingly, it was suspended in sesame oil, and 200 mg. injected into the deltoid muscle. There was no immediate pain although the site of injection in one volunteer became sore by the fifth hour and this persisted through the twenty-fourth hour. Since there was little pain other than that expected from the trauma of injection, 19 male volunteers were injected with 0.8 Gm. of aureotracin suspended in 4.0 ml. of distilled water. Injections were made deep in the gluteal region. The average blood and urine concentrations were determined and are shown in Table IV.

TABLE IV.—AVERAGE AUREOMYCIN AND BACITRACIN CONCENTRATIONS IN BLOOD AND URINE FOLLOWING ADMINISTRATION OF 0.8 GM. OF AUREOTRACIN IN MAN

	Blood		Urine	
Hours	Aureomycin, μg./Ml.	Bacitracin, Units/Ml.	Aureomycin, μg./Ml.	Bacitracin, Units/Ml.
3	1.12	0.20	61	15
21	0.026	0.10	4.9	1.6
26	...	...	3.0	1.0
27	0.026	0.11	...	...
45	0.024	0.14	2.4	0.4
70	...	...	3.0	...

Rather low but significant concentrations of both aureomycin and bacitracin were found in blood and urine from the forty-fifth to the seventieth hours following injection. Examination of the urines showed insignificant traces of albumin in three of the 19 volunteers.

Oral absorption was studied in two volunteer male subjects. Capsules were prepared containing 0.33 Gm. of aureotracin and three capsules were taken daily for five days. Feces and urine specimens were collected daily and bloods were taken at the seventy-fifth hour (three hours after consuming the seventh capsule) and at the one hundred forty-fourth hour (sixteen hours after consuming the last capsule). The serum concentration, calculated as aureomycin, averaged 4 μg./ml. at the seventy-fifth hour and 2 μg./ml. at the one hundred forty-fourth hour. The concentrations of aureomycin in the urine ranged from 68 μg./ml. at the seventieth hour to 22.5 μg./ml. at the one hundred forty-fourth hour. There was no demonstrable effect on the intestinal flora nor was any nausea or discomfort noticed.

SUMMARY

A substance possessing antibacterial properties has been prepared by the reaction of aureomycin and bacitracin in aqueous solution. The resulting material is light to golden yellow in color, amorphous in nature, and highly insoluble in water and ordinary organic solvents. The substance, which has been designated aureotracin, is less nephrotoxic than one would expect from its bacitracin content, has activity against all the organisms ordinarily affected by aureomycin and bacitracin, affords prolonged blood concentration when injected into the animal body, and can be used with safety in man.

The chemical nature of aureotracin is not known although infrared spectrum analysis shows many points in common with aureomycin and bacitracin. The changes in physical characteristics, considered in conjunction with the lowered toxicity to animals, indicate that a substance different from the parent compounds has been formed.

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The Effect of Streptomycin-Bacitracin-Polymyxin Combination, Polymyxin B, and Streptomycin with Glucuronolactone on the Intestinal Flora of Man*

By HENRY WELCH, W. A. RANDALL, and C. W. PRICE

The effect of a combination of streptomycin, bacitracin, and polymyxin B on the intestinal flora is compared with polymyxin alone and with a mixture of streptomycin and glucuronolactone. The streptomycin-bacitracin-polymyxin rapidly eliminated the coliform bacteria and fecal streptococci. Streptomycin with glucuronolactone acted similarly. The polymyxin B was not as effective as the other two preparations. The streptomycin-bacitracin-polymyxin B combination was used with excellent results in the treatment of colitis and chronic diarrhea.

THE oral administration of drugs for modification of the intestinal flora of man has two major objectives. The first one is to reduce the number of microorganisms, especially of certain types, to control infections following gastrointestinal surgery, and the other is to treat infections which are largely confined to the gut. The nonabsorbable sulfonamides were the first to be used for this purpose but had the disadvantage of acting slowly and having too selective an action on the intestinal flora (1). The efficacy of streptomycin in preparing patients for gastrointestinal surgery has been the subject of conflicting reports. Reimann, Price, and Elias (2) reported the appearance of resistant coliforms in patients receiving oral streptomycin. Later Zintel, *et al.* (3), reported sustained reduction of coliform and other groups following oral streptomycin. Further work by Lockwood, *et al.* (4), and Spaulding, *et al.* (1), shows that in most individuals streptomycin may cause a marked reduction in the coliform count for a short period of time (several days), but that later the coliform count returns to normal even though the patient is still under streptomycin treatment. Lockwood, *et al.*, go so far as to conclude that because of its unpredictable effect, its use in preoperative preparation of surgical cases is not to be recommended; Pulaski (5) and Metzger and Shapse (6) consider aureomycin to be unpromising as an intestinal antiseptic; while Dearing and Heilman (7) state that aureomycin is the most effective single substance for reducing the number of intestinal bacteria. Polymyxin [Pulaski (5)] suppresses the coliform group, while the cocci and clostridia were not affected. Chloramphenicol also has some antibacterial effect on the intestinal flora (8).

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EXPERIMENTAL

The present work was begun because of unpublished reports that bacitracin was effective in the treatment of amebic dysentery. Further work (unpublished) showed that this drug is not active against the amoebae themselves, but probably exerts its effect by suppressing the growth of bacteria concerned with the nutrition of amoeba. This finding was confirmed by Bradin and Hansen (9). When given orally to dogs, bacitracin greatly reduced the fecal streptococci and spore-forming anaerobes but had little effect on coliform organisms [Bond, *et al.* (10)]. Preliminary studies in man showed that the addition of streptomycin to bacitracin resulted in a prompt reduction of coliforms, but streptomycin-resistant organisms of this group appeared after two days' treatment. Colonies of these streptomycin-resistant coliform were isolated and tested for their sensitivity to polymyxin B, an antibiotic which is extremely active against many Gram-negative bacilli. All of the streptomycin-resistant strains were found to be sensitive to less than 1.0 μg./ml. of polymyxin B. These studies therefore are concerned with a comparison of a mixture of bacitracin-streptomycin-polymyxin, polymyxin alone, and a drug containing a mixture of streptomycin and glucuronolactone on the intestinal flora of man. Streptomycin with glucuronolactone orally causes a marked reduction in the numbers of coliform bacteria and also other intestinal bacteria. This combination, however, does not prevent the development of strains resistant to streptomycin. The composition and dosage of these drugs are given in Table I.

TABLE I.—COMPOSITION OF DRUGS USED AND DOSAGE SCHEDULES

Drug	Composition of Tablets in Mg.	Dose
Polymyxin	100 (1,000,000 units)	1 tablet once daily
Streptomycin	100	5 tablets 4 times daily
Glucuronolactone	400	
Streptomycin	250	
Bacitracin	5000	1 tablet 4 times daily
Polymyxin	20 (200,000 units)	

The three preparations were given orally to groups of six normal male subjects for a period of ten days. Medication was then discontinued for eight days, resumed for the following six days, and then again discontinued. The fecal flora was studied throughout the entire period including three additional days after the medication was discontinued the second time. Daily stool specimens were collected, an aliquot weighed, and the moist stool diluted 1:10 with distilled water. The coliforms were estimated by making decimal dilutions in distilled water and transferring to lactose broth. The production of acid and gas was considered presumptive evidence of the presence of coliform bacteria. All tubes showing acid and gas were streaked on eosin methylene blue agar plates for confirmation. *Enterococci* were estimated by transferring the water dilutions to sodium azide broth. The sodium azide broth tubes showing either acid and/or growth without acid production were subcultured onto eosin methylene blue agar plates for partial confirmation and confirmed by inspection of the colonies, staining, and microscopic examination. At frequent intervals, subcultures were made from colonies of the coliform and fecal streptococci and their resistance to bacitracin, streptomycin, and polymyxin determined.

The effect of the three preparations on the coliform organisms is shown in Table II.

The streptomycin-bactracin-polymyxin combination and the streptomycin-glucuronolactone produced a rapid fall in numbers of coliform organisms. These organisms were eliminated by the fourth day and remained so for the ensuing period of medication. The fall in numbers was more rapid with the mixture of streptomycin-bacitracin-polymyxin than with the combination of streptomycin-glucuronolactone, especially during the second period of medication. The coliforms were markedly reduced by polymyxin B but were not wholly eliminated.

When the streptomycin-bacitracin-polymyxin combination was discontinued, no coliform bacteria could be demonstrated for three days but thereafter increased to 50,000,000/Gm. by the end of the eighth day. The same was essentially true for the streptomycin-gluronolactone mixture except that small numbers of coliforms made their appearance during the three days following cessation of treatment. Thereafter the numbers increased to the same degree as was obtained with the streptomycin-bacitracin-polymyxin combination. With polymyxin B, the coliform count increased more rapidly than with the other two preparations but by the eighth day without drug, the coliform count did not greatly exceed the count obtained with streptomycin-bacitracin-polymyxin or streptomycin-glucuronolactone. When the preparations were given again, the coliform count of the subjects given streptomycin-bacitracin-polymyxin promptly dropped to zero, while with streptomycin-glucuronolactone, although there was an initial drop to 2,000,000/Gm., the coliforms were not eliminated until the sixth day. After the second course of medication was completed, the coliform count began to return to its former level with all three preparations but rose at a faster rate with streptomycin-bacitracin-polymyxin and with polymyxin B than with streptomycin-glucuronolactone.

The effect on the fecal streptococci is shown in Table III.

The count dropped sharply with the streptomycin-bacitracin-polymyxin mixture and remained so during the entire course of the experiment, including the periods when no drug was given. During the first ten days of streptomycin-glucuronolactone administration, the count fluctuated considerably, resulting in a higher average count than was obtained with the streptomycin-bacitracin-polymyxin combination. Thereafter, the count remained low. The same was true to a greater extent with polymyxin B which had no effect on the fecal streptococci during the first ten days of treatment, but following cessation of drug the count dropped markedly and remained so for the rest of the experiment. It should be pointed out that the fecal streptococci form a very small percentage of the total intestinal flora.

The coliform bacteria and fecal streptococci isolated during the course of these studies were tested for their sensitivity to streptomycin, bacitracin, and polymyxin. A total of approximately 500 strains of coliform bacteria and 200 fecal streptococci were tested. The results are shown in Table IV, in which are tabulated the average resistance before treatment, the average resistance and the range of sensitivity during the rest of the experiment, including the periods during which medication was discontinued.

TABLE II.—THE EFFECT OF A STREPTOMYCIN-BACITRACIN-POLYMYXIN B COMBINATION: STREPTOMYCIN-GLUCURONOLACTONE AND POLYMYXIN B ON THE INTESTINAL FLORA OF MAN: EFFECT ON THE COLIFORM BACTERIA

	Average No. Bacteria/Gm. Wet Feces		
Administration of Drug	Streptomycin-Bacitracin-Polymyxin B	Streptomycin-Glucuron-olactone	Polymyxin B
None—Control	350,000,000	350,000,000	350,000,000
Administered 3 days	720,000	12,260,000	2,800,000
Administered 10 days	0	0	567,000
Discontinued 3 days	0	1,200	7,700,000
Discontinued 8 days	50,000,000	49,200,000	53,000,000
Second course:			
Administered 2 days	0	2,000,000	20,000,000
Administered 3 days	0	2,000,000	2,000,000
Administered 6 days	0	0	40,000
Discontinued 3 days	1,750,000	5,625	2,150,000

Six subjects in each group. Control—average 18 subjects.

TABLE III.—THE EFFECT OF A STREPTOMYCIN-BACITRACIN-POLYMYXIN B COMBINATION: STREPTOMYCIN-GLUCURONOLACTONE AND POLYMYXIN B ON THE INTESTINAL FLORA OF MAN: EFFECT ON FECAL STREPTOCOCCI

Administration of Drug	Average No. Bacteria/Gm. Feces		
	Streptomycin-Bacitracin-Polymyxin B	Streptomycin-Glucuronolactone	Polymyxin B
None—Control	49,000	49,000	49,000
First course:			
Administered 10 days	370	2,000	44,800
Discontinued 8 days	850	250	450
Second course:			
Administered 6 days	150	160	265
Discontinued 3 days	270	70	240

Six subjects in each group. Control—average of 18 subjects

TABLE IV.—SENSITIVITY OF COLIFORM BACTERIA AND FECAL STREPTOCOCCI TO STREPTOMYCIN, BACITRACIN, AND POLYMYXIN BEFORE AND FOLLOWING TREATMENT WITH STREPTOMYCIN-BACITRACIN-POLYMYXIN COMBINATION, STREPTOMYCIN WITH GLUCURONOLACTONE, AND POLYMYXIN B[a]

Time in Relation to Consumption of Drug	Organisms from Subjects Treated with Streptomycin-Bacitracin-Polymyxin — Sensitive to			Organisms from Subjects Treated with Streptomycin-Glucuronolactone — Sensitive to			Organisms from Subjects Treated with Polymyxin B — Sensitive to		
	Sm.[b]	Bac.[c]	Poly.[d]	Sm.	Bac.	Poly.	Sm.	Bac.	Poly.
				(Coliforms)					
Before	8	100	3	8	100	3	8	100	6
After:									
Average	77	110	1.7	270	250	1	11	85	295
Range	8–280	90–150	1–3	100–500	100–500	1	8–18	70–100	20–560
				(Fecal Streptococci)					
Before	45	4	1000	45	4	1000	45	4	1000
After:									
Average	31	3	1000	105	2.5	1000	25	3	1000
Range	15–40	1–4	1000	40–180	1–7	1000	10–50	1–4	1000

[a] Minimal inhibitory concentration in μg./ml. of streptomycin and polymyxin and units of bacitracin.
[b] Sm. = streptomycin. [c] Bac. = bacitracin. [d] Poly. = polymyxin B.

Before treatment, as was to be expected, the coliform bacteria were relatively sensitive to streptomycin and polymyxin and resistant to bacitracin. Following treatment with the two combinations containing streptomycin, the average resistance of the coliform bacteria to streptomycin markedly increased to a point where it was no more effective against these organisms than bacitracin. In the subjects receiving polymyxin B alone, the sensitivity of the isolated coliforms to streptomycin during and after medication was about the same as noted before treatment. It is of interest that the coliforms isolated from the subjects receiving the combination of streptomycin-bacitracin-polymyxin were highly sensitive to polymyxin, while strains from subjects receiving polymyxin alone increased in resistance to this drug from 6 μg. to 295 μg. Under treatment with the combination of streptomycin-bacitracin-polymyxin B, the fecal streptococci did not increase in resistance to streptomycin or bacitracin. With the streptomycin plus glucuronolactone treated subjects, the fecal streptococci definitely increased in their resistance to streptomycin. With all three regimens, the fecal streptococci were quite resistant to polymyxin and sensitive to bacitracin. It is apparent that with any preparation containing streptomycin, one can anticipate the development of coliform bacteria resistant to this drug. The addition of polymyxin to the mixture of streptomycin and bacitracin inhibited the growth of these streptomycin-resistant coliforms, although it does not apparently prevent their development.

The streptomycin-bacitracin-polymyxin combination has been used in 16 cases of gastrointestinal dysfunction. Since none of these three antibiotics is absorbed from the gut in any appreciable quantity, no systemic toxicity was anticipated nor was any observed. No systematic effort to treat diseases of known etiology was made in this study. In most cases the drug was given to physicians at their request for patients in which all other methods of treatment had failed to effect a cure. The cases treated fall into two categories. Four cases of colitis of from two to ten years' duration were treated with excellent results in all cases. The duration of treatment varied from one to three weeks, each patient receiving one tablet four times daily. Twelve cases of chronic diarrhea of undetermined etiology from one-half to fifteen years' duration were similarly treated with excellent results (cure) in eleven patients and marked improvement in the twelfth. It thus appears that this drug may have a definite place in the treatment of certain types of gastrointestinal disease.

DISCUSSION

The effect of various drugs on the intestinal flora has been extensively studied by numerous investigators. The multiplicity of techniques used makes comparisons of results of different workers difficult. If one type of organism, such as *E. coli*, is chosen as an index of the efficacy of a drug, comparisons may be readily made, but when several groups of organ-

isms are included, the evaluation becomes much more difficult.

In this study, all of the subjects under test were on essentially the same diet (prisoners) so that this factor was largely controlled. The streptomycin-bacitracin-polymyxin and the streptomycin-glucuronolactone were about equally effective in reducing the coliforms and fecal streptococci, while the polymyxin B was somewhat less effective.

In view of the little-known and complex biological equilibrium relationships which exist in the human intestinal tract, the evaluation of drugs by means of bacteriological studies may be misleading, and only clinical trial can prove the worth of a given substance. Although a preliminary screening be made to eliminate worthless compounds, it remains for the clinician to determine the usefulness of a drug or drug mixture in man. Streptomycin-bacitracin-polymyxin tablets have been used in the treatment of intestinal diseases of unknown etiology with promising results.

CONCLUSIONS

1. The oral administration of a combination of streptomycin-bacitracin-polymyxin resulted in the virtual elimination of coliform bacteria from the feces for as long as the drug is given.

2. The same was substantially true for streptomycin plus glucuronolactone although disappearance of the coliform bacteria was not quite so rapid.

3. Polymyxin B alone produced a marked reduction in the coliform count but at no time completely eliminated this group of organisms.

4. The fecal streptococci were greatly reduced by the streptomycin-bacitracin-polymyxin combination, streptomycin plus glucuronolactone, and to a lesser extent with polymyxin B.

5. Resistance studies indicated that neither the streptomycin-bacitracin-polymyxin combination nor streptomycin plus glucuronolactone prevent the development of streptomycin-resistant coliforms, although they are suppressed during treatment.

6. The streptomycin-bacitracin-polymyxin combination proved quite effective in the treatment of colitis and chronic diarrhea.

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Iodine-Propylene Glycol Solutions*,†

By LOUIS GERSHENFELD and BERNARD WITLIN

The results of an investigation relating to the use of propylene glycol as a solvent for iodine in pharmaceutical preparations are reported. Physical and biological data are presented which indicate that a solvent for iodine and sodium iodide consisting of water and propylene glycol possesses several advantages over other vehicles.

AN ALCOHOLIC solution of iodine (1/3 ounce of iodine dissolved in 1/2 pint alcohol) known as tincture of iodine was admitted to the U. S. Pharmacopeia one hundred and twenty-five years ago. In 1840, the second revision of this official compendium deleted this plain tincture and recognized compound tincture of iodine which contained one-half ounce of iodine and one ounce of potassium iodide in a pint of alcohol. This compound tincture was deleted in U. S. P. III and the plain tincture, same formula as above, was again made official. In 1900 the plain tincture containing only iodine in alcohol was finally deleted and the compound tincture containing potassium iodide (5 per cent) and iodine (7 per cent) in alcohol was included. However, the name "tincture" was employed for this latter mixture.

Among the aqueous solutions of iodine were compound solution of iodine U. S. P. II, U. S. P. III, and U. S. P. IV (6 drachms iodine and 1 1/2 troy ounces potassium iodide in 1 pint of distilled water). U. S. P. V recommended the use of 360

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grains of iodine, 1/2 troy ounce of potassium iodide, and 1 pint of distilled water.

In 1830, Lugol recommended the use of varying strengths of iodine and potassium iodide according to the purpose for which such solutions were to be employed (1). The U. S. P. in 1870 adopted one modification of Dr. Lugol's "caustic iodine solution," known as "liquor iodi compositus" or as Lugol's (Lugols die Lösung) and employed chiefly for internal use, as a means of obtaining therapeutic effects of iodine, and also as an antidote for alkaloidal poisons. The Lugol (compound solution of iodine) formula in U. S. P. VI (1880) was prepared with iodine (5 parts), potassium iodide (10 parts), and distilled water (85 parts). In 1905, the U. S. P. VIII contained a preparation known as "liquor iodi compositus" (Lugol's solution). It was described as an aqueous solution containing "Iodine, 5 grammes; Potassium Iodide, 10 grammes; Distilled water to make 100 grammes." This formula is basically the same as used today.

In the compound tinctures and solutions of iodine, sodium iodide has been used since 1940 to replace potassium iodide in the formulas, such as in iodine tincture (U. S. P. XIII) and iodine solution (N. F. VIII), because of the lower toxicity of sodium to tissue than that of potassium (2).

MacDonald and Peck (3) recommended in 1928 the use of isopropyl alcohol (90 per cent) as the solvent for iodine stating that it in itself was antiseptic, possessed penetrative powers, was stable, was nontoxic to the skin, and evaporated readily.

LaWall and Tice (4) conducted a comprehensive study of various solvents to find which would be the most suitable for preparing an antiseptic iodine solution. The solvents included: ethanol, methanol, glycerin, benzin, normal propanol, isopropanol, ethyl ether, chloroform, ethylene glycol, cellosolve, cellosolve acetate, carbitol, propylene glycol, dioxan, diethylene glycol, butyl cellosolve, ethyl acetate, dichlorethyl ether, and ethylene dichloride. All solvents containing the primary alcohol group (CH_2OH) were found to be decomposed by iodine with the formation of hydrogen iodide and the corresponding aldehyde (i.e., ethyl aldehyde from ethanol). It was noted that the addition of sufficient potassium iodide to (theoretically) form KI_3 with the iodine in solution, prevented deterioration regardless of the kind of alcohol used or of the alcoholic concentration (5).

From the standpoint of avoiding irritation, the elimination of alcohol especially from open wounds and in cuts and abrasions has been considered as a desirable factor (6). More recently, Price (7) in re-evaluating ethyl alcohol as a germicide stated that: "Ethyl alcohol should not be used as a disinfectant in wounds or in raw surfaces, for it is painful, it injures the tissues and its antibacterial action is neutralized by proteins present in the wounds." Iodine in aqueous solution accordingly has been highly regarded for use to replace iodine in alcohol or alcohol-water solutions as an antiseptic in open wounds.

The findings of Karns and his collaborators resulted in the introduction of a new aqueous iodine antiseptic solution (N. F. VIII) (8).

Objections, however, have been raised to the use of aqueous solutions:

(*a*) Aqueous solutions do not evaporate as quickly as alcoholic or hydro-alcoholic solutions, a feature desired by some especially on the unbroken skin as in preoperative surgery. Actually, excess quantities of aqueous solutions containing 2 per cent free iodine may be wiped from an area and there will still remain an amount of iodine to exert an antispetic action.

(*b*) Manufacturers have avoided recommending aqueous solutions because of the possibility of freezing in cold areas or during cold weather, accompanied by the cracking of glass containers and the resultant possibility of staining other merchandise adjacent to the cracked package.

PROPYLENE GLYCOL

Propylene glycol was chosen as a possible vehicle or solvent inasmuch as it most satisfactorily overcame the objections raised. Propylene glycol has been thoroughly investigated and reported as being a nontoxic solvent, soluble in water, and possessing a low viscosity, skin penetration, and anti-freeze properties (9–11).

In 1896, Pohl (12), using dogs reported that propylene glycol was apparently completely oxidized in the body.

Lehman and Newman, in 1937 (13, 14) stated that after propylene glycol was absorbed by the body, it was rapidly distributed through all tissues, readily oxidized, and that the concentration in the blood was proportional to the concentration in the body.

Neubauer (15) and Miura (16) believed that propylene glycol was partly excreted in conjugation with glucuronic acid.

The acute toxicity of propylene glycol is less than one-half that of ethyl alcohol (13) and has been reported to be nontoxic when given in drinking water over a long period of time (17).

Van Winkle and Newman (18) found that the daily feeding of 5% propylene glycol as drinking water for five to nine months, corresponding to a daily intake of 5.1 cc./Kg., caused neither toxic effects nor pathological and functional changes of liver and kidneys although the average concentration of propylene glycol in the blood ran as high as

325 mg./100 cc. Lethal doses (LD_{50}'s) of propylene glycol have been reported in the rat as being 13 cc./Kg. intraperitoneally; 28 cc./Kg. subcutaneously; and 28 cc./Kg. orally (19).

There are no reports on the injurious effects from industrial use or other use of propylene glycol (11, 20, 21).

Propylene glycol containing iodine (4–16 mg./Kg./day) has been injected subcutaneously for the *in vivo* iodination of tissue protein without any toxic effects (22).

From all data obtained in the literature and experimentally by us, there is no evidence of toxicity which might mitigate against the use of propylene glycol as a vehicle for iodine to be used as a bactericidal solution for skin surfaces, abrasions, open wounds, or mucous membranes.

Because of these properties, it was decided to investigate iodine solutions prepared with propylene glycol and note their antibacterial activity and other important properties.

EXPERIMENTAL

(*a*) An excess of iodine (10 Gm./100 cc. total volume) was added to propylene glycol and thoroughly triturated at room temperature (25° C.). The free iodine present in the supernatant of this mixture was 1.22% immediately after trituration and 3.5% after standing (forty-eight hours up to one month thereafter).

(*b*) Iodine was dissolved in propylene glycol with the aid of heat at 50° C. This revealed 1.36% free iodine immediately following trituration and 3.5%, forty-eight hours later and thereafter.

All iodine-propylene glycol preparations (which were originally prepared with iodine dissolved in concentrated propylene glycol) mixed in all proportions with distilled water, producing clear solutions. Upon standing, however, slight precipitates of crystals of free iodine resulted in these diluted mixtures.

(*c*) The solubility of iodine in various diluted propylene glycol mixtures was determined as in (*a*).

Iodine (1.49%) was soluble in a mixture of propylene glycol 50 parts by volume and distilled water 50 parts by volume.

Iodine (0.89%) was soluble in a mixture of propylene glycol 25 parts by volume and distilled water 75 parts by volume.

Iodine did not precipitate from these diluted propylene glycol solutions nor upon further dilution of these solutions with distilled water.

(*d*) A ten times concentration (10×) of Lugol's iodine solution (50% free iodine) was diluted to regular strength Lugol's iodine solution (5% free iodine), using propylene glycol and various concentrations of propylene glycol in water as the vehicles.

Concentration Used	Final Concentration in Mixture
Propylene glycol (100%)	5.0% free iodine in propylene glycol (90%)
Propylene glycol (75%)	4.98% free iodine in propylene glycol (67.5%)
Propylene glycol (50%)	4.99% free iodine in propylene glycol (45%)
Propylene glycol (25%)	4.99% free iodine in propylene glycol (22.5%)

All of these solutions when diluted with distilled water did not reveal precipitation of iodine. It should be noted that these solutions contained a soluble iodide (present in Lugol's solution). A soluble iodide was not present in the diluted mixtures mentioned under (*b*).

(*e*) Two per cent free iodine solutions were prepared in propylene glycol with varying amounts of sodium iodide and with varying amounts of potassium iodide to determine the amount of iodide required to stabilize the free iodine in solution in propylene glycol when diluted with distilled water.

PROPYLENE GLYCOL SOLUTIONS

Iodine, % (Wt.)	Iodide, %	Free Iodine, % (By Titration)
2.0	2.4	2.05
2.0	2.0	2.02
2.0	1.5	2.009
2.0	1.0	2.02
2.0	0.5	2.03
2.0	0.25	1.39

Starting with the ratio of iodine (2.0) to iodide (2.4) as employed in the present U. S. P. XIII tincture and N. F. VIII solution, the concentration of iodide was decreased. In all instances identical results were obtained if the sodium iodide was replaced by the potassium iodide.

It is to be noted that the concentration of iodide could be reduced to 0.5% with maintenance of complete solubility of the iodine in the propylene glycol-iodide preparation. Lower concentrations of iodide were unable to dissolve more than 1.4% iodine.

All solutions remained clear, revealed no precipitate upon standing, and did not precipitate upon dilution with distilled water.

(*f*) Based upon the findings obtained in the preceding experiment (*e*), the following iodine-iodide solutions were prepared in varying concentrations of propylene glycol. The percentages of free iodine in the solutions prepared with iodine and sodium iodide in propylene glycol were determined immediately following their preparation and at monthly intervals thereafter, up to one year (Table I).

All solutions by themselves and also upon dilution with distilled water were found to be stable for periods longer than one year and did not yield a precipitate.

TABLE I.—PERCENTAGE OF FREE IODINE IN SOLUTIONS OF IODINE AND SODIUM IODIDE PREPARED IN PROPYLENE GLYCOL

Ingredients Added	Concentration of Propylene Glycol 100%	75%	50%	25%
	% of Free Iodine Present			
Iodine 2% Sodium iodide 2.4%	2.05	2.039	2.045	2.048
Iodine 2% Sodium iodide 2.0%	2.02	2.02	2.017	2.02
Iodine 2% Sodium iodide 1.5%	2.009	2.01	2.01	2.01
Iodine 2% Sodium iodide 1.0%	2.002	2.0	2.001	2.002
Iodine 2% Sodium iodide 0.5%	2.003	2.0	2.002	2.003
Iodine 2% Sodium iodide 0.25%	1.39	1.4	1.39	1.39

(*g*) Bactericidal and bacteriostatic efficiency tests were conducted with all of the solutions prepared as above (*f*) using tincture of iodine U. S. P. XIII and iodine solution N. F. VIII as controls.

The bactericidal efficiencies of all the solutions, with the exception of the 0.25% iodide preparations were identical with that of the 2% tincture (U. S. P.) and 2% solution (N. F.). This was due to the fact that not more than 1.4% iodine was soluble in propylene glycol solutions containing 0.25% iodide.

(*h*) Freezing-point determinations were made on the solutions prepared (*f*), and on propylene glycol controls. The freezing points were as shown in Table II.

TABLE II.—FREEZING POINTS OF IODINE-IODIDE-PROPYLENE GLYCOL SOLUTIONS (° F.)

	Concentration of Propylene Glycol			
	100%	75%	50%	25%
Iodine 2% and Sodium iodide 2.4%	−55	−55	−35	−12
Iodine 2% and Sodium iodide 2.0%	−55	−55	−35	−12
Iodine 2% and Sodium iodide 1.5%	−55	−55	−34	−10
Iodine 2% and Sodium iodide 1.0%	−55	−55	−32	− 8
Iodine 2% and Sodium iodide 0.5%	−55	−55	−29	+ 5
Iodine 2% and Sodium iodide 0.25%	−55	−55	−24	+14
Control (Propylene Glycol)	−55	−55	−24	+14

TOXICITY

Rabbits were repeatedly injected with the propylene glycol iodide solution containing 2% free iodine. All intravenous injections using 1.5 cc. of the solution per kilogram of body weight (containing 30 mg. free iodine per kilogram body weight,) did not prove fatal to any of the animals. Doses of 35 mg. (or more) of free iodine per kilogram body weight killed the animals. Rabbits injected (repeatedly) intraperitoneally with 100 cc. of this solution survived.

Hematological studies on the animals which survived did not reveal marked abnormal changes (findings noted before and after injections).

SUMMARY AND CONCLUSIONS

1. Propylene glycol-iodine solutions were investigated.

2. The solubility of iodine in propylene glycol was determined to be 1.36 per cent immediately following preparation and 3.5 per cent forty-eight hours later and thereafter, tests being conducted at monthly intervals up to one year.

3. Solutions of iodine containing a soluble iodide were prepared with aqueous solutions of propylene glycol as the vehicle.

4. Freezing-point determinations were made on all aqueous solutions of iodine-iodide with propylene glycol.

Solutions of iodine-iodide in propylene glycol (in concentrations above 25 per cent) froze at temperatures below −24° C.

5. With the exception of preparations containing 0.25 per cent iodide, the bactericidal efficiencies of iodine-iodide solutions prepared in aqueous propylene glycol vehicles were identical with the bactericidal efficiencies of tincture of iodine (U. S. P. XIII) and iodine solution (N. F. VIII).

6. Propylene glycol can be employed to lower the freezing point of aqueous solutions of iodine. Propylene glycol-iodine solutions possess an adhesiveness on human skin, which may be desirable.

7. Due to the high viscosity of concentrated propylene glycol, it is recommended that it be used in concentrations of not over 50 per cent or lower. To avoid the possibility of freezing of aqueous iodine solutions, propylene glycol should not be used in concentrations below 25 per cent.

8. Toxicity and hematological studies were made with this solution.

9. For a satisfactory aqueous preparation of free iodine possessing an effective bactericidal efficiency, nonstinging, nonirritating and especially where it is desired that it possess a low freezing point, we suggest the following formula: iodine (2 per cent) and sodium iodide (2.4 per cent) in distilled water containing 25 to 50 per cent propylene glycol.

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The Colorimetric Determination of Various Alkaloids*

By F. J. BANDELIN

A number of alkaloids may be quantitatively precipitated from aqueous solution by ammonium reineckate. The alkaloidal reineckate may be dissolved in acetone and determined colorimetrically at 525 mμ. The method is applicable to samples of from 2 to 10 mg. of the alkaloid or alkaloidal salt and has several advantages over the conventional acid-base titration method. Although not specific for alkaloids, the reaction provides a useful and rapid method of determining small quantities of various alkaloids in pure form. Quaternary ammonium salts and certain heterocyclic amines interfere since these also produce insoluble reineckates. A method for the determination of some official tablets containing alkaloids is given with recovery data and comparison with the official method for these preparations.

THE quantitative determination of exiguous amounts of various alkaloids in pharmaceutical preparations is a constant problem of the analytical control laboratory. The extraction of alkaloids by solvents and the subsequent acid-base titration is time consuming and laborious, and leaves much to be desired in specificity. Ammonium reineckate, although long known as an alkaloidal precipitant, has not been investigated as a quantitative method. In an earlier paper (1) the author described a method for the colorimetric determination of homatropine methyl bromide in which the insoluble reineckate of homatropine methyl bromide dissolved in acetone was utilized as a quantitative colorimetric method. This suggested a general method for alkaloids to be investigated with a view toward developing a rapid, accurate procedure for small quantities of alakloids in pure form. Reineckates of quantities of alkaloids in the range of from 2 to 10 mg. were found suitable and adequate for spectrometric determination by this method. The pure alkaloidal salts used in this investigation which were found to give satisfactory results were: atropine sulfate, quinine sulfate, strychnine sulfate, emetine hydrochloride, hyoscine hydrobromide, cinchonidine sulfate, cinchonine, quinidine sulfate, sparteine sulfate, hydrastine hydrochloride, cocaine hydrochloride, and pilocarpine nitrate.

EXPERIMENTAL

Reagents

(*a*) Ammonium Reineckate Solution—2 Gm. of ammonium reineckate[1] was stirred with 100 ml. of distilled water for ten minutes using a mechanical stirrer. The resulting solution was filtered through a hard filter paper. This solution decomposes on standing and should be prepared fresh daily.

(*b*) Sulfuric Acid—1% solution.

(*c*) Wash Water—2 ml. of solution *a* added to enough distilled water to make 1000 ml.

(*d*) Standard Alkaloid Solution—dissolve 100 mg. of the alkaloidal salt in sufficient 1% sulfuric acid to make 50 ml. Each milliliter of this solution contains 2.0 mg. of the alkaloidal salt.

(*e*) Acetone—reagent grade.

Preparation of Standard Curves

Pipette accurately 1, 2, 3, 4, and 5 ml., respectively, of the standard solution of the alkaloid under determination into each of five 20-ml. beakers. Add sufficient 1% sulfuric acid to each beaker to make the volume 10 ml. Place the beakers in a shallow ice bath and add 3 ml. of ammonium reineckate solution slowly and with gentle rotation of the solution in the beaker. Allow these to stand in an ice bath for thirty minutes to assure complete precipitation. Collect the precipitate by filtering through 15-ml. low form fritted glass crucibles of medium porosity using suction. Wash the precipitates by passing two 5-ml. portions of wash water through the crucible with the aid of gentle suction. Continue to apply suction until all the excess water has been removed from the precipitate.

The precipitates are then dissolved in acetone by passing 2-ml. portions of acetone through the crucibles held by a rubber adapter in a funnel whose stem has been drawn out so that it will fit into the neck of a 10-ml. volumetric flask in which the solution is collected. The acetone is best put through the crucibles with the aid of gentle air pressure applied to the surface of the acetone in the crucible. This may be produced by using a single-hole rubber stopper which fits snugly into the top of the crucible. A glass tube with a rubber hand bulb at the end is inserted through the hole in the stopper and the bulb used to produce gentle pressure in the crucible. All the precipitate is usually dissolved in the first three 2-ml. portions of acetone. The solution in the volumetric flask is held just short of volume and this is made up by the addition of acetone from a pipette.

Spectral transmittance of the alkaloidal reineckates in acetone was found to be identical with homatropine methyl bromide reineckate and with ammonium reineckate (1) when determined with a Coleman Spectrophotometer, Model 14, and indicated optimum transmittance at 525 mμ.

* Received April 26, 1950, from the laboratories of Flint, Eaton & Co., Decatur, Ill.

[1] Eastman Kodak Co., Rochester, N. Y.

Percentage transmittance of the alkaloidal reineckates in acetone at 2-mg. increments in the range of 2 to 10 mg. of alkaloidal salt was determined at 525 mμ. Figure 1 shows a typical transmittance curve, plotted on a semilog scale. In this case strychnine sulfate is used as an example. Curves for other alkaloidal reineckates are similar, varying only in slope.

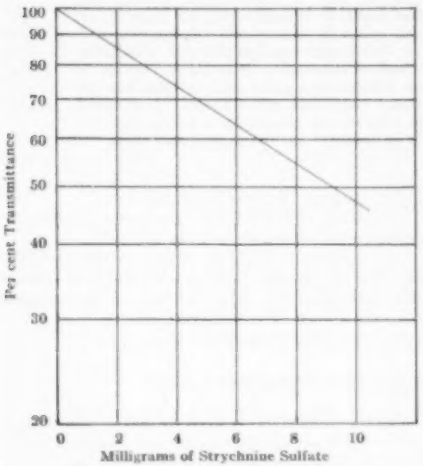

Fig. 1.—Transmittance at 525 mμ.

For the alkaloids investigated, the reineckate method is quite sensitive and at least as accurate as the official method, the much smaller sample required notwithstanding. The usual titration methods using 0.02 *N* acid and alkali, because of the vague and indefinite nature of the indicator end point in these dilute solutions, leave much to be desired in precise determination. For the assay of tablets containing alkaloids, Remington (2) recommends that a minimum of 50 mg. of the alkaloid or alkaloidal salt be used for a determination using acid-base titration. The reineckate method generally required approximately 5 mg. per determination. Since the doses of most of these potent alkaloids are quite small, the differential between the dose and the smallest quantity which can be accurately determined becomes important. Alkaloids having larger doses, such as quinine and quinidine make available considerably more compound per unit dose for analysis. The official assay for these two substances is their gravimetric determination as the alkaloid by the conventional "shake out and weigh method." Here the reineckate method allows accurate and more rapid determination of smaller samples.

The method has extensive application for the determination of pure alkaloids in pharmaceutical preparations. The procedure given may be extended to include tablets as follows:

Method for Tablets

Reduce a sample of not less than 20 tablets to a fine powder in a mortar and extract with a suitable volume of 1% sulfuric acid so that 10 ml. contains approximately 5 mg. of alkaloid. Extraction may be carried out by digesting the powder with the acid on a water bath for thirty minutes, then filtering. The filter should be washed with several small portions of 1% sulfuric acid. Since ordinary tablet ingredients generally do not interfere, filtration is not always necessary since insoluble material is filtered out later, or if soluble in acetone, it gives no color. To the 10-ml. aliquot in a 20-ml. beaker add 3 ml. of ammonium reineckate reagent and allow to stand in an ice bath for thirty minutes. Filter off the precipitate of alkaloid reineckate by passing the solution through a 15-ml. low form sintered glass crucible and wash with two 5-ml. portions of wash water. Dissolve the reineckate in acetone as previously described and read the percentage transmittance at 525 mμ. Determine the amount of alkaloid present by reference to the standard curve for the particular alkaloid or salt.

A number of standard tablet and capsule preparations of various alkaloids were subjected to the reineckate determination and to the conventional methods in use. Tables I to IV give the results obtained in four representative preparations.

DISCUSSION

In the determination of most alkaloids or alkaloidal salts by the acid-base titration method, an aliquot of about 50 mg. of alkaloid is required. By the reineckate method, determinations can be readily and expediently carried out on 5-mg. aliquots with as great or greater accuracy. Deviation from the mean was less with the reineckate method in all cases despite the much smaller sample required.

TABLE I.—1/30 GR. STRYCHNINE SULFATE TABLETS

Reineckate Method Calcd. 5-Mg. Aliquots		Titration Method Calcd. 50-Mg. Aliquots	
Mg. Strychnine Sulfate Found	% Theoretical	Mg. Strychnine Sulfate Found	% Theoretical
5.08	101.6	49.4	98.8
5.12	102.4	49.0	98.0
4.98	99.6	49.6	99.2
4.96	99.2	51.3	102.6
5.14	102.8	50.5	101.0
5.14	102.8	49.8	99.6
5.02	100.4	49.2	98.4
5.06	101.2	49.4	98.8
5.08	101.6	51.6	102.2
4.92	98.4	50.7	101.4

TABLE II.—5 GR. QUININE SULFATE CAPSULES

Reineckate Method Calcd. 5-Mg. Aliquot		Gravimetric Method Calcd. 400-Mg. Aliquot	
Mg. Quinine Sulfate Found	% Theoretical	Mg. Quinine Sulfate Found	% Theoretical
5.08	101.6	404.3	101.1
5.11	102.2	408.7	102.2
5.12	102.4	405.9	101.5
5.06	101.2	412.2	103.0
5.12	102.4	408.3	102.1
5.09	101.8	405.9	101.5
5.10	102.0	403.0	100.8
5.13	102.6	413.2	103.3
5.08	101.6	406.4	101.6
5.10	102.0	409.4	102.5

TABLE III.—1/200 GR. SCOPOLAMINE HYDROBROMIDE HYPODERMIC TABLETS

Reineckate Method Calcd. 5-Mg. Aliquots		Titration Method Calcd. 50-Mg. Aliquots	
Mg. Scopolamine Hydrobromide Found	% Theoretical	Mg. Scopolamine Hydrobromide Found	% Theoretical
4.92	98.4	47.9	95.8
4.97	99.4	48.8	97.6
4.94	98.8	49.1	98.2
4.86	97.2	47.2	94.4
4.93	98.6	48.9	97.8
4.88	97.6	48.2	96.4
4.91	98.2	49.8	99.6
4.94	98.8	48.0	96.0
4.84	96.8	49.6	99.2
4.86	97.2	47.7	95.4

TABLE IV.—1/100 GR. ATROPINE SULFATE HYPODERMIC TABLETS

Reineckate Method Calcd. 5-Mg. Aliquots		Titration Method Calcd. 40-Mg. Aliquots	
Mg. Atropine Sulfate Found	% Theoretical	Mg. Atropine Sulfate Found	% Theoretical
5.15	103.0	41.3	103.2
5.04	100.8	40.8	102.0
4.92	98.4	41.2	103.0
5.12	102.4	39.2	98.0
5.10	102.0	41.9	104.7
4.98	99.6	41.6	104.0
5.08	101.6	42.2	105.5
4.96	99.2	41.8	104.5
5.16	103.2	42.6	106.5
5.13	102.6	40.8	102.0

Although not absolute, there is a certain specificity in the reineckate method in producing a precipitate with various alkaloids since ammonium reineckate produces precipitates only with quaternary ammonium salts, heterocyclic nitrogen compounds, and certain tertiary amines. In this respect the method is more specific than acid-base titration or Kjeldahl determination for nitrogen.

The method is relatively simple and rapid and largely eliminates extraction by solvent partition. The usual tablet or capsule lubricants and excipients do not interfere in the determinations. The color intensity of the alkaloidal reineckates is such that quantities of from 2 to 10 mg. of alkaloid or alkaloidal salt give transmittance values of from 90 to 30% at 525 mμ on a Coleman Spectrophotometer. Photoelectric colorimeters equipped with a filter transmitting at 525 mμ give very satisfactory results.

Starch, talc, and insoluble stearates need not be filtered off since these do not interfere with the precipitation, the development of the color, or the clarity of the acetone solution of the reineckate.

During the course of this investigation it was noted that the various alkaloids gave characteristic precipitates with ammonium reineckate. The difference is apparently due to characteristic crystalline structure of the precipitates, and this suggests the possibility of specific identification by microscopy. At least one such identification using ammonium reineckate has been described (3).

Ephedrine, colchicine, and morphine could not be quantitatively precipitated by ammonium reineckate.

SUMMARY

A number of alkaloids may be determined quantitatively by precipitation from acid solution with ammonium reineckate. After filtration the alkaloidal reineckate is dissolved in acetone and determined colorimetrically at 525 mμ using a spectrophotometer or suitable photoelectric colorimeter. Standard curves may be prepared for the various alkaloids or alkaloidal salts listed. Morphine, ephedrine, and colchicine are not completely precipitated by ammonium reineckate, hence the method is not applicable to them. A method for tablets and capsules is given. Tablets of atropine sulfate, strychnine sulfate, scopolamine hydrobromide, and capsules of quinine sulfate were investigated both by the official and the reineckate method. In all cases the reineckate method was more sensitive and more accurate, with more consistent results on much smaller samples than with the official method.

Although various organic bases other than alkaloids interfere by yielding insoluble reineckates, the limitations conferred upon the method by this analogy are no greater than in the case of the titration method.

The reineckate method may undoubtedly be applied to other alkaloids, outside the scope of this investigation, with corresponding success.

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Effect of Ultraviolet Irradiation on *Escherichia coli* and Its Reversal*

By ROBERTSON PRATT, JEAN DUFRENOY, and GRACE GARDNER†

The mechanism of germicidal action of ultraviolet radiations, although assumed to be through their ionizing and oxidizing effects, is not entirely known. The present paper presents evidence that, in common with other oxidants and some antibiotics, ultraviolet radiations induce a shift to the right in the equilibrium —SH⇌S—S and that the activity of mononucleotidases is impaired concomitantly. Treating bacteria with appropriate agents capable of altering the redox potential or of destroying H_2O_2 markedly improved the survival chances of *Escherichia coli* exposed to ultraviolet radiation.

DIRECT SUNSHINE has long had repute among the laity as an antiseptic. This concept, based on empirical observation, has been applied scientifically and industrially in the development of germicidal lamps which emit ultraviolet radiations, principally with wave length 2537 Å.

In the last decade or two, the mechanism of action of various radiations of short wave length on living cells has acquired new interest. During World War II, the mutagenic properties of certain wave lengths in the ultraviolet and X-ray portions of the spectrum were employed extensively in antibiotic research to produce high-yielding mutant strains of naturally occurring antibiotic-producing microorganisms. In fact, the strain of *Penicillium* used most extensively today for industrial biosynthesis of penicillin is an ultraviolet-induced mutant derived from an X-ray-induced mutant of a "wild" strain of *P. chrysogenum*.

More recently, concern of public health officers over the possibility of induced sterility, cancer, eye cataracts, altered blood count, and other pathologic symptoms, long recognized as occupational hazards of X-ray technicians, has been intensified because of the relatively large number of individuals engaged in atomic research and development and in research involving radioactive isotopes. Currently, the only really effective means of protecting workers from the effects of these short wave radiations seems to be physical shielding. Often this is cumbersome, inconvenient, and expensive.

The present paper is a report on certain prophylactic treatments which tend to protect bacterial cells against the lethal effects of the 2537 Å. line emitted by a cold mercury arc. Understanding of the mechanisms of action of ultraviolet radiations and of chemical methods of combating their effects in relatively simple cells might ultimately lead to better means of protecting man and other animals from their effects and from the more serious effects of certain other short wave radiations.

Irradiation of living organisms or tissues may have multifarious effects, as follows:

(*a*) A bombardment effect, which may induce physical changes in the chromosomes or stereochemical changes in some of their molecules. This bombardment effect is brought into play in the artificial induction of mutations.

(*b*) A heat effect due to absorption of radiant energy. Nucleoproteins are especially sensitive to this effect.

(*c*) An oxidizing effect which arises from ionization. It is largely with this effect and its consequences that the present report is concerned.

It is well known that ultraviolet and other short wave radiations promote formation of ozone from atmospheric oxygen and formation of hydrogen peroxide from the oxygen dissolved in water. They may also induce formation of organic peroxides from various constituents of cells and of nutrient media. One might expect, therefore, that ultraviolet or other similar radiations would induce effects in living tissues similar to those resulting from exposure to oxidants or other agents tending to favor a shift to the right in the equilibrium —SH ⇌ S—S. This is supported by the literature (1).

Although similarity of symptoms does not constitute proof of a common causal reaction, it may be considered suggestive of such a reaction. The similarity of cytochemical effects induced in microorganisms by ultraviolet or by X-ray irradiation, and by certain antibiotics known to

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favor dehydrogenation of —SH groups (2) may signify implication of a common cytochemical denominator. It is significant in this connection that erythema, due to exposure to the rays of the sun or of ultraviolet lamps, has been correlated with increased conversion of sulfhydryl-containing proteins of the skin from the reduced to the oxidized state (3) and that radiation sickness resulting from exposure to X-rays may be alleviated by administration of reduced glutathione or of cysteine (4). Of course, in complex tissues such as the skin, excitation entails vasodilation and other changes in the circulatory system: even pathologic changes, including hemorrhage, may result from increased permeability of the blood vessels. Such secondary complications may be prevented or alleviated to some extent by proper use of rutin (5), vitamin C (5), and possibly vitamin K.

EXPERIMENTAL

Since we were concerned with fundamental cytochemistry in these experiments, for greater convenience we used bacterial cells as test objects instead of histologically more complicated organisms or tissues. Glutathione and other —SH-bearing proteins are no less important in the metabolism of bacteria than in man or other higher animals (6).

Methods.—Broth cultures of *Escherichia coli* (eighteen to twenty-four hours at 37°) were diluted $1:10^8$ in sterile phosphate buffer (*p*H 6.9). Five milliliters of the diluted culture were placed in each of several sterile Petri dishes and exposed for different lengths of time to the radiation from a 20-watt germicidal lamp (Americanaire, Model UVM-15, manufactured by American Sterilizer Co., Erie Pa.) at a distance of 63 cm. This is a "cold" mercury type lamp. The principal ultraviolet emission is at 2537 Å. Immediately following irradiation, the cultures were placed at 2° for one to two hours. Then 10 ml. of melted agar, cooled to 50°, was added to each dish, and the buffer and agar were thoroughly mixed. The cultures were incubated for forty-eight hours at 37°, after which the number of colonies that had developed was recorded.

The agar was rendered suitable for growth of the test organisms by addition of the culture medium described by Kalmanson and Bronfenbrenner (7) slightly modified. The modified medium consisted of: (*a*) $(NH_4)_2HPO_4$, 5 Gm.; succinic acid, 4.5 Gm.; water, 500 ml.; and agar, 10 Gm.; and (*b*) dextrose, 20 Gm.; $MgSO_4 \cdot 7H_2O$, 1 Gm.; K_2SO_4, 1 Gm.; water, 500 ml.; and agar, 10 Gm. Solution *a* was adjusted to *p*H 7.2. The two solutions were then autoclaved separately. When cool, they were mixed in equal proportions and the *p*H was readjusted with sterile NaOH to *p*H 7.0.

Effectiveness of various reagents in nullifying the cidal action of irradiation was determined principally by adding appropriate quantities of the compounds to the buffered cell suspensions before exposure. An equal volume of sterile distilled water was added to the controls. In one experiment the reagent was added after irradiation.

Preliminary experiments showed that exposure for 15 seconds at 63 cm. permitted approximately 40% survival in control cultures (Fig. 1). Since this was a convenient reference base for our work, all results reported are from such exposure.

The temperature of the suspensions did not exceed 27° during irradiation. Therefore, heat may be ruled out of these experiments as a cidal factor.

Results.—Ultraviolet irradiation, inasmuch as it

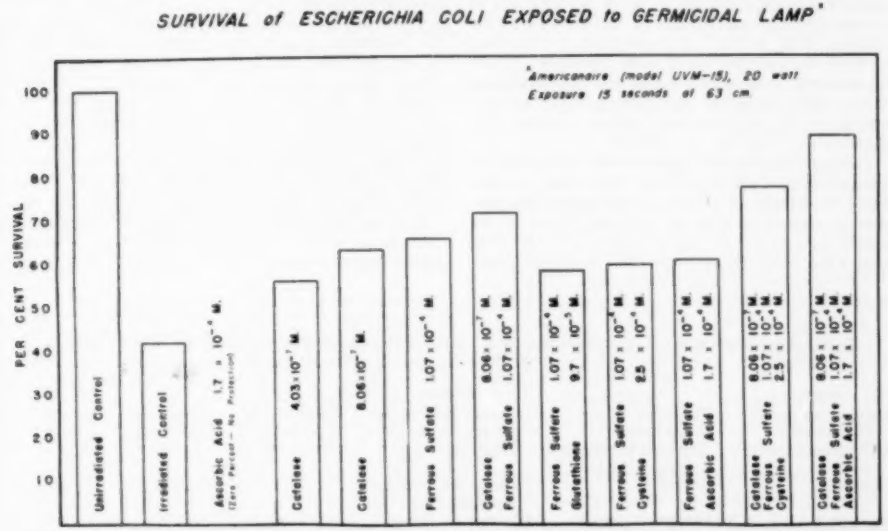

Fig. 1.—Survival of *Escherichia coli* exposed to ultraviolet irradiation when suspended in various solutions. Survival, as indicated by number of colonies developing upon subsequent plating, in the experimental suspensions is expressed as a percentage of survival in unirradiated controls. See text for details.

promotes formation of O_2 from atmospheric O_2 and of H_2O_2 from dissolved O_2, must tax to the utmost the ability of the test organisms to destroy H_2O_2 and maintain the concentration below that which inhibits growth and division.

The experiments reported below were designed to test the possibility of canceling the oxidizing or dehydrogenating effects of ultraviolet radiation by (*a*) making available to the organisms a surplus of reduced glutathione or cysteine which might serve as a reservoir for the natural supplies and help to prevent their depletion below the critical threshold level; (*b*) augmenting the normal biosynthetic supply of catalase which is capable of destroying H_2O_2; and (*c*) adding ferrous sulfate, known to have a peroxidasic effect, or of other reducing agents that might be expected to share with the test organisms the task of reducing hydrogen peroxide or other peroxides.

Although staining reactions indicated that several of the reagents conferred some measure of cytochemical protection on cells irradiated in their presence, the protection, as judged by number of colonies that developed upon incubation, was not statistically significant except when catalase or ferrous sulfate was present. The percentage survival afforded by the various single components and mixtures that provided statistically significant protection is shown in Fig. 1. It may be seen that whereas there was approximately 60% mortality (41.2% survival) in populations irradiated without benefit of chemical protection, it was possible, by use of appropriate single agents or combinations of them, to significantly reduce the mortality. The most effective protection in these experiments was provided by an appropriate mixture of catalase, ferrous sulfate, and ascorbic acid, which reduced the mortality to less than 15% (88.3% survival).

Statistical treatment of the data according to the methods of Fisher (8) for "the significance of the mean of a unique sample" and for "comparison of two means" showed that the protection afforded by catalase (4.03×10^{-7} *M*) could not be expected to occur from chance alone more than twice in 100 experiments. Analysis showed that the likelihood of the results observed with ferrous sulfate or with ferrous sulfate and various admixtures arising from chance alone was even more remote.

Various cytochemical reagents can be used to advantage to give an estimate of the relative abundance or activity of certain important cell constituents in microorganisms at different phases of the growth cycle (2). Several of these, and the techniques for using them, have been described previously (9, 10). For *E. coli* these may be summarized as shown in Table I.

Application of these reagents showed that the effects of ultraviolet irradiation in the absence of added catalase or of a suitable reducing agent are (*a*) to accelerate dehydrogenation of —SH groups and deplete the organisms and medium of reducing groups (as detected notably by cacotheline); and (*b*) to promote formation of H_2O_2 in excess of the amount which the normally biosynthesized catalase can decompose. This results in enhancement of the over-all activity of oxidase (as tested by the Thydi reagent) and of peroxides, as evidenced by change in the orange color of indigosol green solution to purple and then green.

Ultraviolet irradiation, like many other agents promoting dehydrogenation of —SH groups, also blocks mononucleotidase activity and, therefore, results in accumulation of mononucleotides which may be seen as deep blue deposits when the cells are treated with a solution of trypan blue.

DISCUSSION

Several explanations for the survival data plotted in Fig. 1 are plausible. First, it might be argued that several of the agents used are capable of absorbing in the ultraviolet region and that, therefore, their action was primarily a physical shielding or screening effect which prevented most of the radiation from reaching the organisms. Several facts militate against this argument, however, so that screening certainly could not have been a major factor.

The strongest argument against the screening hypothesis is afforded by the fact that in an experiment in which the catalase-ferrous sulfate-ascorbic acid mixture was added *after* irradiation some reversal of the detrimental effects of ultraviolet was noted. The average number of colonies developed from the irradiated controls was 50.5 ± 1.5, whereas 70.1 ± 1.8 colonies developed upon subsequent plating of an irradiated suspension to which the above-mentioned mixture was added following irradiation. These figures represent 43.4% and 62.1%, respectively, of the value for the nonirradiated control. In nonirradiated cultures, the chemicals did not improve growth of the organisms over the controls. It may be concluded, therefore, that in the experimental series the benefit accruing from use of the several reagents was indeed due to reversal of the effects of irradiation and cannot be attributed merely to action of the compounds as growth-promoting substances.

TABLE I.—TESTS FOR CHARACTERISTICS OF *E. coli* AT DIFFERENT PHASES OF THE GROWTH CYCLE

Compound	Reagent	Reaction	Phase of Growth Cycle		
			Lag	Log	Stationary
Ribonucleic acids	Toluidine blue	Specific adsorption	Synthesized	Used up	Scanty
—SH proteins	Cacotheline	Orange → purple	Synthesized	Used up	Scanty
Dehydrogenases	Triphenyltetrazolium chloride	Colorless → red	Synthesized	Active	Sluggish
Oxidases	Thydi	Formation of indophenol blue	.....	Active	Evident
Peroxides	Indigosol green[a]	Orange → green	.....	Active	Evident
Catalase	.....	O_2 evolution	Synthesized	Active	Lacking

[a] Indigosol green IB is available from Durand and Hugenin, Basel, Switzerland.

It will be noted that addition of the catalase-ferrous sulfate-ascorbic acid mixture after irradiation yielded approximately 20% improvement over the irradiated controls whereas when added before irradiation the benefit was greater. However, this difference cannot be fully accounted for, if at all, by physical screening of the ultraviolet rays.

Absorption curves, determined by means of a Beckman Ultraviolet Spectrophotometer, showed that even ascorbic acid, which of the agents used, has the strongest absorption in the 2537 Å. region, transmitted at least 70% of the incident radiation. Data for this and the other single components used are shown in Table II. Furthermore, it should be pointed out that ascorbic acid alone not only failed to afford protection, but was actually detrimental. There were no survivors (judging from subsequent lack of growth) among cells irradiated in the presence of ascorbic acid. This may have been due to some of the decomposition products of ascorbic acid, which is destroyed extremely rapidly when exposed to 2537 Å. radiation. Moreover, due to the rapid destruction of ascorbic acid by ultraviolet, any screening effect must have been a continuously diminishing function.[1]

It appears from the figures in Table II that the screening effect of the solutions used could not fully account for the survival values observed.

Two of the most obvious explanations to account for the survival values shown in Fig. 1 are (*a*) that the effective reagents acted by destroying H_2O_2 or other peroxides formed in the medium or in the cell fluid, and (*b*) that reducing agents, by being oxidized themselves, partially prevented peroxide formation. In either event they could serve to poise the redox potential at a level that would permit maintenance of the balance —SH⇌S—S within the range that is compatible with life and growth.

No special precautions were taken to maintain the iron sulfate in the reduced state prior to use. Consequently, although fresh solutions were employed, traces of ferric sulfate were also present. However, this would be advantageous from the standpoint of decomposing H_2O_2, since small amounts of Fe^{+++} ion tend to catalyze decomposition of hydrogen peroxide by Fe^{++}.

Additional evidence favoring the chemical interaction hypothesis over mere physical screening is found in the report that solutions containing oxygen acceptors prevent the oxidation of strychnine to genostrychnine under the influence of ionizing radiations (11). Sodium nitrite (but not nitrate), ferrous (but not ferric) sulfate, and stannous (not stannic) chloride were effective in preventing the oxidation.

TABLE II.—TRANSMISSION OF INCIDENT 2537 Å. RADIATION THROUGH 1 MM. PATH LENGTH OF AQUEOUS SOLUTIONS AT *p*H 6.0–7.0

Solution	Concentration	% Transmission
Ascorbic Acid	1.70×10^{-4} *M*	70
Ferrous Sulfate	1.07×10^{-4} *M*	85
Catalase	8.06×10^{-7} *M*	98

Other evidence also points to the importance of oxidation effects. For example, boosting the natural supply of reduced glutathione or of cysteine, which, under the oxidizing agency of ultraviolet, one would expect to be oxidized to the S—S form, is effective prophylactically and therapeutically in radiation sickness (4, 12), and mere subsequent exposure to visible light, which readily decomposes hydrogen peroxide, effectively counteracts the deleterious action of prior ultraviolet exposure in some microorganisms (13–15), in sea urchin sperms (16, 17), and in bacteriophage (18).

SUMMARY

The effect of various compounds, in low concentrations, on survival of ultraviolet-irradiated *Escherichia coli*, as evidenced by ability of the cells to grow was determined under conditions that permitted about 40 per cent survival in unprotected irradiated controls. Catalase and ferrous sulfate each afforded some measure of protection, as shown by higher survival percentages in cultures irradiated in their presence. A mixture of catalase, ferrous sulfate, and ascorbic acid, which permitted about 85 per cent survival, was most effective of the treatments studied. This appeared to be an anomalous situation, since irradiation in the presence of ascorbic acid alone was detrimental. The deleterious effect of ascorbic acid alone, in the presence of ultraviolet, however, was probably due to toxic decomposition products. Calculations based on measurement of absorption curves showed that the protective action cannot be fully accounted for on the basis of physical screening. It seems likely that agents which chemically vitiate the ionizing or oxidizing effects of ultraviolet radiation without production of toxic products are beneficial in protecting cells from the effects of ultraviolet irradiation.

It is evident that the results noted cannot be ascribed merely to physical screening through ultraviolet absorption by the compounds, since the mixture of catalase, ferrous sulfate, and ascorbic acid, while not acting as a growth stimulator for nonirradiated cells, did reverse the growth-depressing action of ultraviolet whether added before or after irradiation.

[1] In fact, it is quite likely that destruction was nearly, if not entirely, complete after exposure for fifteen seconds to the 2537 Å. line. This idea is supported by the fact that ascorbic acid alone afforded no protection whatsoever to irradiated organisms. In view of this observation, it is difficult to account for the beneficial effect of ascorbic acid in the catalase-ferrous sulfate mixture. This mixture alone permitted about 72% survival, but when ascorbic acid was added nearly 88% of the irradiated organisms survived and grew. Discussion of the complex chemical phenomena involved in such a system is beyond the scope of this report. Spectrophotometric determinations showed, however, that catalase significantly protects ascorbic acid, even in the presence of ferrous and ferric ions, against decomposition by ultraviolet radiation. Thus it may be that enough active ascorbic acid was present under these conditions to exert some screening effect, although calculations based on absorption data showed that such an action could not account for more than a few per cent increase. It could not account for the 15% higher protective value of the catalase-ferrous sulfate-ascorbic acid mixture. Moreover, the results of experiments in which the agents were added after irradiation should be kept in mind.

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Collaborative Studies on Thiamine Mononitrate in Pharmaceutical Preparations*

By JOHN C. BIRD† and ROBERT S. SHELTON‡

The results of a series of collaborative studies on the relative stabilities of thiamine mononitrate and thiamine hydrochloride are reported. In a wide variety of pharmaceutical preparations considerable deterioration of the nitrate occurred except in tablets in which the loss in potency was negligible. Liquid preparations showed significant losses and changes in color especially at a temperature of 40°.

SINCE the commercial production of thiamine hydrochloride, this synthetic vitamin has been used in practically every pharmaceutical form, *viz.*, tablets, capsules, syrups, elixirs, parenterals, etc. Some difficulty has been experienced by most manufacturers as regards its stability in certain of these products. This deterioration seems to be based largely upon *p*H of the environment. Liquid preparations have proved among the most troublesome, since injectable solutions of thiamine hydrochloride frequently develop a yellowish-to-brown tint on storage, while turbidities coupled with threadlike "floaters" or dark particles appear. Until recently, when special grades of thiamine for parenteral use were offered by manufacturers, many pharmaceutical houses experienced such deteriorative effects which in some quarters came to be regarded somewhat as inevitable and inherent in the thiamine molecule. Perhaps as a result of the above, thiamine mononitrate has lately been made available as an alternative to the now well-known hydrochloride.

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A report of collaborative work organized by the authors and presented before the Eastern and Midwestern Scientific Sections of the American Pharmaceutical Manufacturers' Association in February, 1950.
† Chairman, Eastern Scientific Section, A.P.M.A.
‡ Chairman, Midwestern Scientific Section, A.P.M.A.

METHOD

In view of the above, the authors organized a collaborative study of this new salt, and cooperation of the laboratories of six well-known pharmaceutical houses was enlisted. Five representative products were made to provide each laboratory with sufficient material for the year's series of tests upon each.

The items prepared were as follows:

(*a*) Plain uncoated tablets containing 25 mg. thiamine mononitrate.
(*b*) Soft gelatin capsules plain, 10 mg. thiamine mononitrate each.
(*c*) Soft gelatin capsules containing tartaric acid to give *p*H 3.8 in water, 10 mg. thiamine mononitrate each.
(*d*) Elixir, using aromatic elixir U. S. P. adjusted to *p*H 4 (5 mg. thiamine mononitrate/cc.).
(*e*) Sterile solution adjusted to *p*H 3.8, 5-cc. ampuls containing 50 mg./cc.

The *p*H adjustments were made with nitric acid to avoid any possibility of confusion with, or contamination by, the hydrochloride.

Products were dispatched so that each participant would receive them at approximately the same time. Four dates were set so that at definite periods each product was assayed at the same time by each laboratory. Samples were kept in the dark in boxes at two temperature levels, room temperature and 40°. Assays were made at the outset, after three months, six months, and one year. Figures used in the tables, etc., were averages of the six separate determinations on each preparation.

The U. S. P. XIII thiochrome method of assay was used, omitting the enzymatic digestion, adsorption, and elution steps. In calculating the thiamine mononitrate content, the difference in mol. wt. between the hydrochloride and nitrate necessitated the use of the factor 0.9706 for conversion of apparent thiamine hydrochloride to mononitrate.

Table I shows the general results which are reproduced graphically in Figs. 1 and 2. Losses are shown in parentheses.

TABLE I.—THIAMINE MONONITRATE STABILITIES IN VARIOUS PREPARATIONS AFTER ONE YEAR AT ROOM TEMPERATURE AND AT 40°

Items	As Made, Mg.	Found at Start	After One Year RT	After One Year 40°
Tablets	25	25.32	25.07	24.63
Capsules (*p*H 6.0)	10	9.84	9.17	9.07
Capsules (*p*H 3.8)	10	10.04	9.21	8.12
Elixir (*p*H 4.0)	5	5.03	4.29	2.93
Parenteral (*p*H 3.8)	50	47.88	44.51	41.93

DISCUSSION

It will be noted that the tablets proved to be the most stable preparation. In other words, the mononitrate if kept "dry" (*i.e.*, in powder form as sold) does not appear to deteriorate to any significant extent. The soft gelatin capsules—both "plain" and acidic—lost about the same amount (6–8%) at room temperature, but at 40° the capsules containing tartaric acid lost considerably more (about 2½ times) than the others. In the case of thiamine hydrochloride capsules the reverse seems to be true, for it is usual to lower the *p*H of such capsules by purposely adding tartaric acid to formulations when possible.

The elixir made the worst showing, losing almost 15% at RT and nearly 42% at 40°. Moreover, samples turned a darker yellow shade although generally maintaining clarity.

The ampulled preparations assayed low at the start, some destruction apparently having taken place during manufacture, perhaps in the heat sterilizing process. Loss, however, was calculated on the basis of 50 mg./cc. actually used, and amounted to 7% and 12% (approximately) in the RT and 40° specimens, respectively. These solutions were yellow in color, which in the case of the 40° samples, turned deeper as time progressed. After twelve months at 40° some showed a turbidity together with a yellowish precipitate. Similar behavior has of course been noted with regard to the hydrochloride but usually without such deep color change. It might be that adjustment of *p*H with nitric acid might be conducive to slight yellowing for manufacturers' data concerning the mononitrate states that "ampul solutions adjusted to *p*H 4.0 *with hydrochloric acid* have shown no precipitation and only slight discoloration after storage in the dark at room temperature for twenty-one months." Incidentally, the parenteral solutions consisted solely of the mononitrate adjusted with nitric acid, with

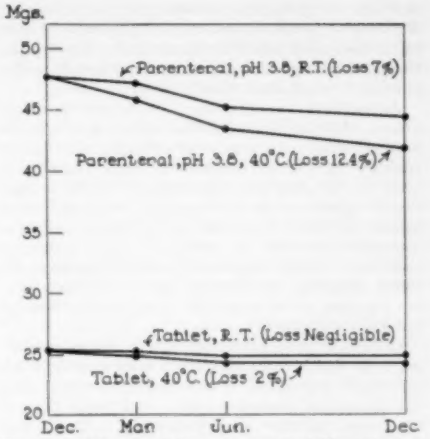

Fig. 1.—Thiamine Mononitrate Stability During One Year.

no other additions. The recommended use of hydrochloric acid seems to us somewhat surprising, for the purpose of the new compound would presumably seem to be to avoid some of the inherent and undesirable characteristics of the hydrochloride.

It must be borne in mind that the above series of tests were upon preparations of the mononitrate only. No comparative figures using the hydrochloride alone were obtained as it was thought that most laboratories using thiamine in their products would already possess ample records on stabilities. The data given, therefore, might be used by way of comparison with such records. In presence of materials such as metallic salts, other vitamins, liver extracts, and powders, the figures might be different. Taub (1), for example, reporting on the

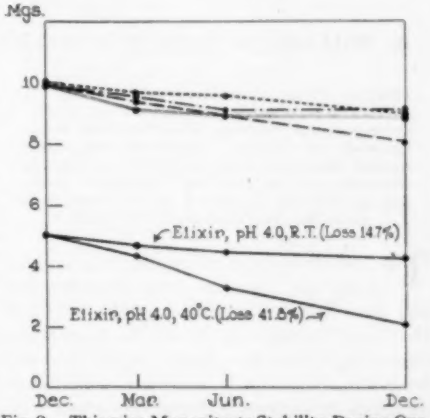

Fig. 2.—Thiamine Mononitrate Stability During One Year.

— · — · — Plain Capsules, *p*H 6.0, R.T. (Loss 6.8%)
——— Plain Capsules, *p*H 6.0, 40° C. (Loss 7.8%)
- - - - - Acid Capsules, *p*H 3.8, R.T. (Loss 8.0%)
– – – – Acid Capsules, *p*H 3.8, 40° C. (Loss 19.4%)

stability of thiamine mononitrate in parenteral B complex preparations and in presence of iron salts, concluded that the nitrate under these conditions was somewhat superior to the grade of hydrochloride he used at the time.

According to manufacturers' statements the mononitrate possesses several initial advantages over the hydrochloride. On a molecular weight and moisture content basis it represents 3% more vitamin B_1 activity. It is of lower hygroscopicity and is practically odorless. It is, however, more difficultly soluble in water than the hydrochloride and considerable agitation is necessary when preparing solutions. Other properties such as biological activity, toxicity, stability to light, oxidation, etc., are reported to be essentially the same as those of the hydrochloride.

In collaborative work such as this, some interesting facts which otherwise might pass unnoticed are sometimes uncovered. For example, we found that one particular laboratory was consistently high in its assays, another consistently low. The others were extraordinarily close which speaks well for accuracy of techniques and reproducibility of the assay method. In the case of the "high" and "low" laboratories, it might be that apparatus or techniques are in need of check, and attention will consequently be drawn to our observation.

Thanks are due to our collaborators as follows: E. B. Vliet, Abbott Laboratories; M. E. Avery, Lederle Laboratories Division, American Cyanamid Company; S. H. Fox, Gelatin Products Division, R. P. Scherer Corporation; H. R. Kreider, Wm. S. Merrell Company; L. M. Roeg, Merck & Company; and C. W. Sondern, White Laboratories.

SUMMARY

1. With a view to comparing thiamine hydrochloride with a more recently available salt, thiamine mononitrate, a collaborative series of stability tests was carried out over a period of one year using the nitrate in a variety of pharmaceutical preparations.

2. Deterioration of the nitrate took place in all with the exception of the tablets in which loss of potency was negligible.

3. Liquid preparations showed significant losses—particularly the elixir. Losses were accentuated at 40°, while color changes were considerable.

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Pyrexia in Rabbits Following the Injection of Filtrates of Typical Mold Cultures*

By WILLIAM D. HARKNESS, WALKER L. LOVING, and F. ALLEN HODGES

Filtrates of mold cultures representing 31 species which were propagated on a dextrose-peptone medium were injected intravenously in rabbits. Significant elevations in body temperature varying over a wide range were observed for 16 of the 31 species. Pyrogen production appeared to be dependent upon the composition of the media.

THE febrile reaction which sometimes follows intravenous injection of certain fluids has long been a disturbing factor to the physician (1, 2), and its cause was obscure for many years. One of the earliest investigators in this field was Wechselmann (3) who observed temperature elevations in patients following venoclysis with arsphenamine solutions for the treatment of syphilis.

The production of pyrogenic substances by bacteria has been established by several workers. Hort and Penfold (4) emphasized the danger of using contaminated injectables. They reported that intravenous injections of isotonic sodium chloride solution often elicited a febrile response when the material injected had been filtered through Berkefeld candles to remove bacteria.

Later, investigational work concerning the pyrogenic potentialities of the schizomycetes was carried out by Bourne and Seibert (5), McClosky, *et al.* (6), Welch, *et al.* (7), Robinson and Flusser (8), Probey and Pittman (9), and others. They employed the filtrates of numerous species of bacteria, some of which produced fever in rabbits. The widespread property

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among various microbes to provoke a thermal response is now well recognized. Co Tui and Schrift (10) further demonstrated the ability of bacteria to produce pyrogen and suggested the probability that mold contaminants in parenterals may produce a temperature rise following intravenous injection. Wylie and Todd (11) gave additional attention to various genera and species of microorganisms and reported that, of those tested by them, only bacteria were capable of producing substances which elevate body temperature.

Since there is reason to believe that organisms other than bacteria may elaborate fever-producing elements, the object of the experiments herein reported was to study the pyrogenicity of culture filtrates of several species of mold grown in two different substrates.

MATERIALS AND METHODS

Thirty-one species of molds[1] representing 18 genera were studied. All cultivations were at room temperature. Appropriate inoculation of a suspension of mycelial hyphae and/or spores was made into 500-cc. Erlenmeyer flasks containing 200 cc. of either of the following:

(*a*) Sabouraud liquid medium
- 40 Gm. dextrose
- 10 Gm. peptone
- 1000 cc. distilled water

(*b*) Czapek liquid medium (12)
- 2.0 Gm. sodium nitrate
- 1.0 Gm. monopotassium phosphate
- 0.5 Gm. potassium chloride
- 0.5 Gm. magnesium sulfate
- 0.01 Gm. ferrous sulfate
- 30.0 Gm. sucrose
- 1000 cc. distilled water

Sabouraud liquid nutrient broth was used in the cultivation of all the mold species under investigation. In addition, ten of these species were grown in Czapek liquid substrate.

The incubation period varied from twelve to thirty days with tests being done as soon as good growth was obtained and the surface of the substrate covered with a mycelial "mat." The length of incubation was apparently not the limiting factor in pyrogen production, since positive and negative results were well randomized among the varying ages of the cultures.

Following the growth period, the liquid cultures were examined microscopically to assure freedom from contamination, then centrifuged until clear and the supernatant liquid filtered through Berkefeld (N) candles. In each case the filtrate was tested for pyrogens by the U. S. P. XIII method (13). For evaluation of the results the official definition of a positive assay was observed and in addition the average maximum temperature rises of the groups of three rabbits were considered and these figures are presented.

[1] Appreciation is expressed to Elwyn T. Reese, Quartermaster Depot, U. S. Army Service Forces, Philadelphia, Pa., who supplied 15 of the mold species used in this study.

As control, sterile 200-cc. aliquots of each of the various lots of media were held for the same period as the portions inoculated with mold. These were then filtered and examined for pyrogens as in the case of the mold culture filtrates.

RESULTS AND DISCUSSION

Filtrates representing 16 of the 31 species propagated in Sabouraud liquid medium were found to give a positive U. S. P. XIII assay for pyrogen (see Table I). Assays of cultures of *Aspergillus sydowi*, *Popularia sphaerosperma*, and *Memnoniella echinata* were borderline in that only one of the three rabbits injected in each case showed a temperature increase as great as 0.6°.

In contrast to the above results which indicated the presence of pyrogen were those obtained when the various lots of sterile media were injected. In no instance did the animals injected with the uninoculated control media respond with a temperature rise as high as 0.6°. The average temperature increase in 24 animals used for this purpose was 0.21°.

TABLE I.—RESULTS OF INJECTION OF FILTRATES OF MOLD CULTURES AT DOSAGES OF 10 CC. PER KG. BODY WEIGHT

Organism	Average Maximum Temperature Rise in °C. Sabouraud Liquid	Czapek Liquid
Blakeslea trispora	2.07	...
Fusarium (species unknown)	1.83	0.10
Aspergillus flavus	1.73	0.13
Gliomastix convoluta	1.43	0.23
Fusarium roseum	1.40	0.27
Aspergillus niger	1.37	0.20
Aspergillus oryzae	1.27	0
Cephalothecium roseum	1.17	0
Stachybotrys atra	1.17	..
Aspergillus ochraceous	1.10	0.10
Alternaria tenuis	0.87	..
Trichoderma viride	0.80	..
Curvularia lunata	0.77	..
Aspergillus versicolor	0.63	..
Popularia sphaerosperma	0.63[a]	0.07
Gliocladium roseum	0.63	..
Penicillium (species unknown)	0.60	..
Memnoniella echinata	0.53[a]	..
Phoma pigmentivora	0.40	..
Aspergillus sydowi	0.37[a]	..
Cladosporium herbarum	0.37	..
Rhizopus nigricans	0.27	..
Thielavia sepedonium	0.27	..
Aspergillus fumigatus	0.23	..
Aspergillus terreus	0.20	..
Fusarium (species unknown)	0.20	..
Myrothecium verrucaria	0.20 Toxic[b]	..
Aspergillus repens	0.03	..
Alternaria solani	0.03	0.17
Penicillium citrinum	.. Toxic[c]	..
Aspergillus luchuensis	.. Toxic[d]	..

[a] One rabbit in three gave temperature rise of 0.6° or more.
[b] All three animals in state of muscular weakness immediately following injection and all died within twenty-four hours.
[c] All three animals exhibited increased heart rate and respiration, pupillary dilation, and died within five minutes after injection.
[d] All three animals responded with severe muscular tremor, collapse, and death within five minutes after injection.

Since it is well known (14) that pyrogens of bacterial origin can be removed from solutions by adsorption on the Seitz filter pad, tests were conducted to determine if similar results could be obtained with the pyrogens produced by molds. Berkefeld filtrates of six species which gave positive tests for pyrogens were passed through single-layer Seitz filter pads and reassayed. In each case there was a marked reduction of pyrogenicity.

Penicillium citrinum grown in Sabouraud liquid medium was lethal to rabbits. The toxic nature of this species has been observed by previous investigators including Ambrose and DeEds (15) who have presented an exhaustive study of its active principle, citrinin.

Ten mold species cultivated in Czapek medium did not produce a pyrogenic response when filtrates of these cultures were injected, although eight of these had given positive results following cultivation in the Sabouraud medium (see Table I). It appears that nutrients should be given special attention when studies regarding the sources of pyrogen are conducted.

Wylie and Todd (11), following investigation of several mold species, presented data supporting the conclusion that pyrogen was not produced by the species studied. Their medium of choice was Czapek-Dox, which is identical to Czapek liquid medium. As already mentioned, our results with this substrate were also negative.

SUMMARY

1. A pyrogenic response in rabbits was obtained with culture filtrates prepared from 16 of 31 species of mold investigated.

2. Significant temperature increases were obtained only when the molds were cultured in Sabouraud's liquid medium containing dextrose and peptone. Negative results were obtained when ten of the mold species were cultivated in Czapek's liquid medium, which contains inorganic nitrogen, sucrose, and inorganic salts.

3. Marked variation in temperature response was observed for different species of molds belonging to the same generic group.

4. As control, pyrogen assays on each of the several separate lots of the media employed gave entirely negative results.

5. Seitz filtration eliminated or greatly reduced the pyrogenicity of the cultures.

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American Association for the Advancement of Science—Pharmacy Subsection Meeting

The next meeting of the American Association for the Advancement of Science will be held in Cleveland on December 26–31, 1950. The program of the Pharmacy Subsection of the Medical Sciences is being made up. Four sessions of this Subsection will be held as follows: Cleveland Public Auditorium, Parlor C, Thursday, December 28, 9:30 a. m. and 2:00 p. m.; Friday, December 29, 9:30 a. m. and 2:00 p. m. The Hollenden Hotel has been designated as headquarters for Section Np.

Authors who wish to present papers before the Subsection are requested to send in titles of papers *as soon as possible.* All titles for inclusion in the printed program should be in the hands of Glenn L. Jenkins, Chairman; Subsection Np; Purdue University, School of Pharmacy; Lafayette, Indiana, by *September 15.*

Standard Tolerances for Pharmaceutical Compounding. A Basis for Their Establishment. III. Capsules*

By SAMUEL W. GOLDSTEIN†

THE PROCEDURES employed in these studies were described and discussed in the two preceding papers in this series (1). The data obtained with sets of filled capsules that were prepared extemporaneously by many licensed pharmacists at their prescription counters are considered in this paper.

The tolerance or permissible deviation for the average unit weight in a set of capsules is determined from the average of the standard deviations obtained for all the "selected" sets of capsules in each series. The average standard deviation (S.D.) in grains is converted to the coefficient of variation expressed in per cent. The coefficient of variation is multiplied by the factor 1.73 to obtain the calculated tolerance. The assigned tolerance is obtained by reference to a scale which increases by increments of 2.5.

The S.D. for the requested total weight is determined from the squared deviations from the theoretical total weight in all the "selected" sets of capsules in the series. The calculated tolerance is determined from the S.D. by conversion to the coefficient of variation and use of the factor 1.73.

The tolerance for the extreme unit deviation in any set of capsules can be reasonably proposed as twice the assigned average unit tolerance. This is generally allowed in tablet regulations.

EXPERIMENTAL

All the purchased capsules contained a single ingredient. They were tested by determining the weight of the contents of each individual capsule. Lee (2) determined content weights by weighing the removed powder and by weighing the filled capsule against an average empty capsule weight as a tare. He reported more accurate results by the latter method and, although both procedures yielded results below the theoretical weight, the emptying procedure gave slightly lower weights.

In this study the capsules were purchased at many drugstores and represent many different batches from the manufacturers of gelatin capsules. The wide variation in the empty capsule weights precludes the use of an average weight as a tare. The slower and more tedious and trying procedure which was used requires the transfer of the capsule contents, as completely as possible, to a tared watch glass which is weighed on an analytical balance. This procedure would be expected to yield low results because of the difficulty in completely removing the capsule contents. Nevertheless, the number of capsules showing plus deviations is surprisingly high. Of the 61 selected sets of 5-gr. quinine sulfate capsules, 31 sets show a plus deviation from the total weight. Of the 83 sets of 5-gr. phenacetin capsules, 33 sets show plus deviations from the total weight. Of the 60 selected sets of 3-gr. aspirin capsules, 32 sets show plus deviations. The four disqualified sets of 3-gr. aspirin capsules show plus deviations of 25–40%.

The first series contains 83 sets of 5-gr. phenacetin capsules, each set consisting of 10 capsules. The data are summarized in Table I. The calculated tolerance for the total weight of each set of capsules is ±6.00%. The calculated tolerance for the average unit weight is ±7.36%.

The second series contains 61 selected sets of 5-gr. quinine sulfate capsules, each set consisting of 6 capsules. The data are summarized in Table I. The calculated tolerance for the total weight of each set of capsules is ±7.63%. The calculated tolerance for the average unit weight is ±9.40%.

The third series contains 60 selected sets of 3-gr. aspirin capsules, each set consisting of 10 capsules. The data are summarized in Table I. The calculated tolerance for the total weight of each set of capsules is ±10.93%. The calculated tolerance for the average unit weight is ±11.83%.

DISCUSSION AND CONCLUSIONS

The two series of 5-gr. capsules yield determined tolerances showing some differences. The differences might probably be due to the use of sets of 10 capsules and sets of 6 capsules, or to additional manipulative difficulties with the quinine sulfate. A limited check using 5 "selected" sets of 10 capsules of quinine sulfate did not support the idea that the differences were caused by the use of sets of 6 capsules. Tolerances of 6.73% for the total weight, and of 9.42% for the average unit weight were obtained. Nevertheless, it is clearly demonstrated that tolerances of ±7.5% for the total weight, of ±10% for the average unit weight, and of ±20% for extreme unit deviation are sufficiently reasonable for single component 5-gr. capsules. Strict application of the assigned standards allows acceptance of 93% of the phenacetin capsule sets and 85% of the selected quinine sulfate capsule sets.

* Received March 6, 1950, from the Bureau of Chemistry, Maryland State Health Department, Baltimore.
† Pharmaceutical Chemist, Maryland State Health Department.

TABLE I.—SUMMARY OF ACCUMULATED DATA

A. Standard Deviations in Grains of Individual Sets of Capsules

Capsule	No. of Sets Purchased	Lowest S.D.	S.D. of Lower Quartile	S.D. of Median	S.D. of Upper Quartile	Highest S.D.
Phenacetin 5 gr.	83	0.0608	0.1389	0.1830	0.2963	0.7976
Quin. Sulf. 5 gr.	66	0.0194	0.1586	0.2362	0.4014	1.5230[a]
Aspirin 3 gr.	64	0.0458	0.1096	0.1911	0.2971	1.2631[b]

Highest S.D.'s in the selected series are [a] 0.6068 and [b] 0.5263 gr.

B. Deviations in Grains from Total Set Weights

Capsule	No. of Sets Purchased	Total Wt. Requested, Gr.	Lowest Dev.	Dev. of Lower Quartile	Dev. of Median	Dev. of Upper Quartile	Highest Dev.
Phenacetin 5 gr.	83	50	0.03	0.66	1.13	1.90	7.92
Quin. Sulf. 5 gr.	66	30	0.06	0.36	0.89	1.60	9.23[a]
Aspirin 3 gr.	64	30	0.00	0.61	1.35	2.27	11.98[b]

Highest deviations in the selected series are [a] 2.78 and [b] 4.82 gr.

C. Sets with Units Exceeding Indicated Deviations

Capsule	Total No. of Sets	Per Cent Deviation Exceeded: 10	15	17.5	20	22.5	25
Phenacetin 5 gr.	83	23	8	5	3	2	2
Quin. Sulf. 5 gr.	66	30	12	9	7	5	3
Quin. Sulf. 5 gr.	61[a]	24	8	5	3	1	0
Aspirin 3 gr.	64	34	20	14	10	8	5
Aspirin 3 gr.	60[a]	30	16	10	6	4	1

[a] Selected sets. These are used in tolerance determinations.

The data obtained with the series of 3-gr. aspirin capsules indicate that tolerances of ±10% for the total weight, of ±12.5% for the average unit weight, and of ±25% for extreme unit deviation might be reasonable for single component 3-gr. capsules. Strict application of the assigned standards allows acceptance of 87% of the selected sets.

The system of tolerance determination utilized in these studies appears to yield reasonable and equitable tolerances when applied to any class of extemporaneously compounded pharmaceuticals. The present report on capsules indicates that the system is applicable to this class of preparations. The further studies necessary to determine standards of tolerance for the wide range of capsules of different weights and numbers of ingredients are clearly indicated. Reasonably large series of sets of capsules should be used in studies of this nature in order to obtain dependable data. Further studies should be carried out to determine reasonable tolerances for one-, two-, and three-ingredient capsules, having total unit weights of 10 gr. or more, 5–10 gr., 3–5 gr., and less than 3 gr.

SUMMARY

1. Tolerances are determined for single-ingredient, 5- and 3-gr. capsules.

2. Further evidence is presented that use of "selective sampling" and 1.73 standard deviations yield reasonable and equitable tolerances for extemporaneously compounded pharmaceutical preparations.

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Standard Tolerances for Pharmaceutical Compounding. A Basis for Their Establishment. V. Divided Powders*

By SAMUEL W. GOLDSTEIN†

A RECENT STUDY of 13,125 prescriptions (1) shows that divided powders account for 2.9 per cent of all compounded prescriptions. This indicates that about 290,000 sets of divided powders are compounded annually in the United States. The present study was undertaken to determine whether reasonable and equitable standards of tolerance can be determined for extemporaneously compounded divided powders.

Reasonable and equitable standards of tolerance for extemporaneously compounded liquid preparations have been obtained by determination of 1.73 standard deviations of series of "selectively sampled" prescription-counter products (2). This method of tolerance determination has been successfully applied to ointments, capsules, and hand-made pills (3–5). The same procedure has now been utilized with powders that were prepared by practicing pharmacists for a known state drug inspector.

EXPERIMENTAL

Forty-five sets of ten powders containing calomel 32.4 mg. and sodium bicarbonate 324 mg. per powder were tested. The contents of each powder were transferred completely to a tared watch glass and weighed on an analytical balance. The combined contents of the ten powders in each set were treated with water to dissolve the sodium bicarbonate. The filtered bicarbonate solution and the washed residue of calomel on the paper were assayed by official procedures. The data obtained from the analytical results are given in Table I.

The difference between the total weight of powder found in each set of 10 powders and the requested total weight is squared. The average squared value is reduced to the square root to obtain the standard deviation from the total weight. The other standard deviations are determined by squaring the determined individual unit deviations from the theoretical quantity in each set of powders. The individual unit weights of the separate ingredients are obtained by application of the determined percentage concentration to the weight of each powder. Sets of powders and individual powders that showed deviations greater than 30% were omitted from determinations of tolerances. Three sets of powders that deviated more than ±30% from the total requested weight were omitted completely.

TABLE I.—DATA FROM CALOMEL AND SODIUM BICARBONATE POWDERS

Set No.	Squared Deviation from Requested Weight, 3.564 Gm.	Standard Deviation from Theoretical Weight, 356 Mg. per Powder	Standard Deviation of HgCl from 32.4 Mg. per Powder	Standard Deviation of $NaHCO_3$ from 324 Mg. per Powder
1	0.0112	11.99	2.38	11.02
2	0.0182	29.51	2.81	28.58
3	0.0001	6.91	1.56	6.89
4	0.0083	9.54	1.11	12.27
5	0.0433	24.60	4.07	21.62
6	0.0062	17.56	2.36	16.33
7	0.0066	25.02	2.42	23.50
8	0.0058	7.30	3.02	6.17
9	0.0023	9.90	0.87	9.30
10	0.0185	15.39	1.15	13.98
11	0.0159	16.87	2.38	15.39
12	0.0001	3.73	0.50	2.97
13	0.0100	13.85	2.89	11.87
14	0.0012	36.83	3.71	35.46
15	0.2324	55.70	3.33	52.82
16	0.0005	4.17	0.53	4.85
17	0.0795	32.93	3.72	27.45
18	0.1473	55.75	6.99	51.80
19	0.0069	9.24	2.73	10.92
20	0.0640	26.68	0.89	24.83
21	0.3916	63.55	3.96	60.00
22	0.0164	27.44	3.80	25.31
23	0.0114	11.57	3.36	8.99
24	0.0110	12.84	0.65	11.10
25	0.0071	9.05	3.27	7.14
26	0.3785	68.00	5.63	59.05
27	0.0001	1.82	1.37	1.84
28	0.0149	29.71	3.17	25.61
29	0.0276	33.62	19.95[a]	28.03
30	0.0045	21.90	5.27	18.69
31	0.0645	26.97	1.65	24.92
32	0.6839	84.63	3.35	86.85
33	0.0117	20.53	1.83	18.82
34	0.0061	7.51	2.54	5.55
35	0.0077	12.41	0.89	11.77
36	0.0076	11.49	1.95	10.97
37	0.0104	15.62	3.78	13.74
38	0.0006	6.71	2.82	5.76
39	0.0077	12.45	0.91	11.85
40	0.0005	44.70	7.70	40.41
41	0.0012	8.96	2.42	7.22
42	0.0190	22.40	5.67	18.74

Standard deviation from requested total weight per set, 0.2371 Gm.

Average standard deviation from requested unit weight, 23.02 mg.

Average standard deviation of HgCl from theoretical unit weight, 2.81 mg.

Average standard deviation of $NaHCO_3$ from theoretical unit weight, 21.20 mg.

[a] Omitted from determination of average standard deviation.

* Received August 25, 1949, from the Bureau of Chemistry, Maryland State Health Department, Baltimore.

† Pharmaceutical Chemist, Maryland State Health Department.

Conversion of the respective standard deviation and average standard deviations to the equivalent coefficients of variation and multiplication by the ratio 1.73 yields the following determined limits of permissible error: (*a*) requested total weight (3.564 Gm.) ±11.5%; (*b*) average unit weight (356 mg.) ±11.2%; (*c*) average unit weight of calomel (32.4 mg.) ±15.0%; (*d*) average unit weight of sodium bicarbonate (324 mg.) ±11.3%. Assignment of values from a scale that varies by increments of 2.5% yields the respective tolerances: (*a*) ±12.5%; (*b*) ±12.5%; (*c*) ±15.0%; (*d*) ±12.5%. Values for (*a*), (*b*), and (*d*) are raised to 12.5 because they exceed 10.0 by more than 1.0. The assigned tolerances for these three divisions coincide with the tolerances that would be assigned by the system determined for liquid preparations. That system is based upon the total weight of the medicinal ingredient requested.

Four of the 42 "selected" sets of powders do not meet the ±12.5% tolerance for requested total weight. Six sets do not meet the assigned tolerances for the average unit weight per powder; the average unit weight of calomel; and the average unit weight of sodium bicarbonate. Acceptance of 92% of the first division, and of 86% of the other three divisions by 1.73 standard deviations shows that with this type of preparation, as with the other types tested, the samples follow a normal statistical curve.

DISCUSSION

The products utilized in this study are composed of ingredients that can be considered as typical for extemporaneously prepared divided powders. Mordell's study (1) showed that 13% of all the compounded divided powders contained calomel as one of the ingredients, and 5% contained both calomel and sodium bicarbonate. Thirty-nine per cent of all the compounded divided powders contained sodium bicarbonate, and 13% contained sodium bicarbonate and only one other ingredient.

Three of the 11 powders in the N. F. contain sodium bicarbonate, and the only two powders that have official assays for ingredient concentration are among these three. The N. F. monographs give quantities for the preparation of 1000 Gm. of all powders. The U. S. P. recognizes only compound effervescent powders, and one of the two component sets of powders contains sodium bicarbonate. Amounts and directions for the preparation of 12 sets of powders are given in the monograph. This is a carry-over from the days when these powders were extemporaneously compounded to a large extent.

The results of this study indicate that the tolerances determined for powders follow closely the tolerance-by-weight pattern established with liquid prescription-counter products. Until further work is reported, reasonable tolerances for powders can be assigned according to the following weight classification:

Group I. Weight 5 Gm. or more. Tolerance ±10%.
Group II. Weight 2.25 to 4.99 Gm. Tolerance ±12.5%.
Group III. Weight 0.5 to 2.24 Gm. Tolerance ±15%.
Group IV. Weight 0.49 Gm. or less. Tolerance ±17.5%.

The weights utilized are the requested weights of the individual and the combined ingredients in the whole set of powders.

SUMMARY

1. The system of tolerance determination for extemporaneously compounded pharmaceuticals which utilizes "selective sampling" and 1.73 standard deviations has been applied to calomel (32.4 mg.) and sodium bicarbonate (324 mg.) powders.

2. The assigned tolerances are: Requested total weight (3.56 Gm.) ±12.5 per cent; average unit weight per powder ±12.5 per cent; average unit weight of calomel ±15.0 per cent; average unit weight of sodium bicarbonate ±12.5 per cent.

3. The determined tolerances follow the tolerance-by-weight pattern established with liquid preparations, and that system can be applied tentatively to other extemporaneously compounded divided powders.

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Significance of Variations in Responses of Five Species of Perfused Hearts to Common Drugs*

By BRADFORD N. CRAVER and ANNE CAMERON†

The effects of a series of common drugs upon the perfused hearts of five species of animals have been studied and the results are reported. The significance of the results for a mechanism of drug action from two standpoints is discussed.

THE study reported herein was suggested by the diametrically opposite effects of the cardiotonic members of a series of aryloxyacetamidines upon the perfused hearts of cats and rabbits (1). In the hope of gaining some insight into the mechanism of action of these drugs in particular, and of cardiac metabolism in general, the species of hearts studied were extended to include not only the cat and rabbit, but also the guinea pig, hamster, and rat. In addition to the drugs mentioned, those listed in Table I were studied in the hope the qualitative and quantitative variations in the pattern of the responses of the five species would contribute to the purpose of this study.

METHOD

The apparatus and procedure employed herein have been previously described (2). The volume of each injection was kept below 0.5 ml. for the largest hearts, feline and rabbit, and below 0.1 ml. for the smallest. It was injected fairly rapidly into the capillary terminating in the perfusion stream just above the aortic cannula. Since that capillary contains 0.8 ml. the drug did not actually become available to the heart until the bulb on the medicine dropper was slowly squeezed over a five- to fifteen-second interval. The shorter interval was used with the largest hearts and the longer with the smallest. The bulb was held tightly squeezed for five to fifteen seconds, and then slowly permitted to refill. This procedure not only washes all drug out of the capillary, but also leaves the kymographically recorded myocardial contractions unaltered when saline alone is injected. After each injection 10 ml. of the otherwise-recycled perfusion fluid was rejected. For each drug the minimally effective dose (M. E. D.) was determined for each species of heart and represents the average of at least two separate experiments with each species. With each drug the approximate M. E. D. was determined in a preliminary experiment. Figure 1, depicting the variations of the five species in their responses to ephedrine sulfate, illustrates the degree of change, whether inhibitory or stimulatory, defined as an M. E. D. Table I should not only be a valuable reference for teaching demonstrations, but may suggest to others ideas of mechanism of action that have eluded us.

In all experiments the coronary flow was recorded with an automatic flowmeter which has already been described (2). Since this records increments of 10.0 ml., the drugs in the minimal doses employed would not be expected perceptibly to influence the flow. Had smaller increments of flow been measured, slight changes after some drugs might have been observed. The exceptions to this will be discussed under results.

RESULTS

The results have been summarized in Table I. It is clear that the responses of any heart to a drug will depend upon the concentration of the drug reaching it and that in turn will depend upon several variables: the concentration of the drug injected, the rate of injection, the rate of flow of perfusion fluid through the heart,[1] and the completeness of mixing of the solution of the drug with the perfusion fluid prior to its entrance into the coronary arteries. The impact of these variables may differ somewhat depending upon the peculiarities in the structure of the particular apparatus employed for the perfusion.

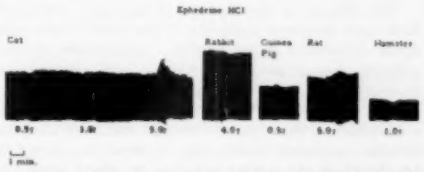

Fig. 1. -Effect of ephedrine hydrochloride on the five species of hearts. Since this drug exerted a positive inotropic effect on some, and a negative inotropic effect on the others, it illustrates the degree of change, whether inhibitory or stimulatory, defined as a minimally effective dose (M. E. D.).

A correction may be made for those in any given apparatus by checking one or two of the doses in the table and applying the resultant rough correction factor to the others. Since all figures in Table I were obtained under the same conditions with the same apparatus, they may be validly compared, which is not possible for the innumerable data widely scattered in the literature. The figures quoted are probably correct within a factor of 5; i.e., normal biological variations should not lead to doses greater than five times those noted or less than 20% of those noted.

* Received May 8, 1950, from the Research Department, Division of Macrobiology, Ciba Pharmaceutical Products, Inc., Summit, N. J.
† With the technical assistance of Mary Laskey.

[1] Average coronary flows in ml./min. for the 5 species were: cat, 10–25; guinea pig, 5; hamster, 2; rabbit, 10–25; rat, 5.

TABLE I.—ACTIONS OF COMMON DRUGS ON FIVE SPECIES OF PERFUSED HEARTS (DOSES IN μg. UNLESS OTHERWISE INDICATED)

Drug	Cat	Rabbit	Guinea Pig	Rat	Hamster
Acetylcholine	1.0	0.1	0.005–0.01	0.005	0.005
	−	+	+ −	−	−
Aminophylline	100–200	100–200	10–50	5.0	3.5
	+ −	−	+	−	−
Antistine	20–50	20–50	5.0	5–10	10.0
	−	−	−	−	−
Atropine[a]	20	20–50	10–20	1–2	0.5
	−	−	−	−	−
Digifolin	0.025–0.05 c. u.	0.05 c. u.	0.025 c. u.	0.05–0.1 c. u.	0.1 c. u.
	+	+	+	+	+
Doryl	0.5	0.25–0.5	0.001–0.005	0.002	0.002
	−	+	+ −	−	−
Ephedrine	0.5–1.0	4.0	0.5	2.0–5.0	1.0–2.0
	+	−	+	+	−
Epinephrine	0.019	0.01–0.02	0.01–0.02	0.01–0.02	0.02
	+	+	+	+	+
Histamine	Variable	0.2	0.005–0.01	5.0	10–20
	−	−	+ −	−	+ −
Mecholyl	1.0	0.5–1.0	0.002–0.005	0.005	0.005
	−	+	+	−	−
Papaverine	50–100	50–100	2.0–5.0	5–10	0.5
	+	−	+	+	−
Pituitrin (S)	Variable	0.002 I. U.	0.002–0.004 I. U.	0.001–0.002 I. U.	0.01–0.02 I. U.
		+	+	+	−
Priscoline	5–20	200–300	5.0	10.0	10–20
	+	−	+	−	−
Privine	20–50	10	0.5	1.0	0.5–1.0
	−	−	−	−	−
Pyribenzamine	20–50	50	5.0	5–10	10
	−	−	−	−	−
Trasentine	20–100	50–100	5–10	3.0	5–10
	−	−	−	−	−

[a] These doses mildly inhibit cardiac action, but lower doses (2–3 μg.) block effective doses of Doryl and Mecholyl.
+ = Augmentation of amplitude of contractions.
− = Diminution of amplitude of contractions.

The effects of the various drugs upon coronary flow have not been included in the table, since only three influenced it perceptibly: papaverine hydrochloride, histamine hydrochloride, and epinephrine hydrochloride. In the higher minimal doses, i.e., 100 γ, papaverine hydrochloride increased significantly the coronary flow of the cat and rabbit hearts. Similar increases occasionally occurred in respect to the heart of the guinea pig, but no effect upon the hearts of the rat and hamster was observed, probably because the increments of coronary flow measured were relatively so large in respect to the rate of flow through these two species of hearts. Doses of histamine hydrochloride of the order of 3–5 γ invariably increased the coronary flow in the hearts of the cat, rabbit, and guinea pig. It did not produce discernible changes in the coronary flow of the rat and hamster hearts. The minimal dose of epinephrine increased the flow of the hamster heart, but did not alter the flow of the other four species of hearts. In respect to the cat heart, higher doses caused a decreased coronary flow, but these were doses giving a marked stimulation. Doses giving moderate stimulation did not influence the flow.

It seemed improbable that the low doses of the drugs employed, especially because they were so slowly injected, could have influenced the activity of the heart purely by changes in *p*H induced. To settle this question various doses of 0.1 *N* hydrochloric acid and 0.1 *N* sodium hydroxide were injected into the cannula. After 100 γ of the alkali no significant change appeared but as the dose was increased to 500 γ increasing stimulation appeared which even after the highest dose was less than a 10% increase in the amplitude of the myocardial contractions. Even 100 γ of the acid caused slight changes in the amplitude. Significant decreases in the amplitude of the contractions occurred as the dose was augmented to 500 γ (maximum decrease ca. 20%). Neither the acid nor the alkali influenced the coronary flow since their actions were so brief. These results would suggest that amine hydrochlorides given in doses of less than 100–200 γ probably did not exert their effects purely from changes in *p*H since they are less than 1% as acid as hydrochloric acid. Since no strongly alkaline salts were employed the possible effects of upper *p*H ranges can be ignored.

A few of the drugs had quite different effects upon some of the hearts when given in doses several times the M. E. D.'s. This was especially true for the parasympathomimetics that in doses from 5–10 times the M. E. D.'s caused the anticipated cardiac inhibition. Doses of Pituitrin of the order of 10–100 times the M. E. D.'s caused marked inhibition of the amplitude of myocardial contractions in respect to the hearts of the rabbit and rat.

Ten times the M. E. D. of ephedrine inhibited the heart of the guinea pig. In respect to the cat heart, an analogous dose caused, as usual, marked stimulation which shortly yielded to a marked inhibition. With these exceptions higher doses of the drugs caused quantitative but not qualitative changes in the responses.

DISCUSSION

If one makes the plausible assumption that the final contractile mechanism of each of these five species of hearts is the same, i.e., a reversible alteration in the structure of a protein with the energy supplied by adenosine triphosphate (3), then certain conclusions would appear reasonable in the light of the data presented:

1. Epinephrine in essentially identical doses exerted the same effect on all five species of hearts. This seemingly identical sensitivity is doubtless partly an illusion, since no corrections were made in respect to any of the drugs for the difference in weight of the hearts perfused. Nonetheless, this remarkable similarity would suggest that epinephrine either acts directly upon or very close to the final contractile mechanism, or, as a second alternative, that it acts on the link of an enzymatic chain of unknown length, but so basic phylogenetically that it is shared by all the species studied despite other metabolic differences.

2. The failure of ephedrine to act alike on all species is further support for the contention of Ahlquist (4) and of Shaw, Papper, and Rovenstine (5) that the hypothesis this drug acts by conserving endogenous epinephrine or sympathin is no longer tenable.

3. Digifolin acts on all five species of hearts in remarkably similar manner both quantitatively and qualitatively. This would suggest it acts directly upon the contractile mechanism of the muscle cell or very close thereto. Certain other implications as to the mechanism of action of the cardiac glycosides will be presented in another report.

4. The antihistaminics, 2-[benzylphenylaminomethyl]-imidazoline (Antistine) and N,N-dimethyl-N′-benzyl-N′-(α-pyridyl)-ethylenediamine (Pyribenzamine), were essentially identical in their effects upon the five hearts. These two antihistaminics and those mentioned below were employed in the form of their commonly available salts. Subsequently the following antihistaminics were shown to act upon the feline heart like Antistine and Pyribenzamine: N,N-dimethyl-N′-phenyl-N′-benzylethylenediamine (Antergan), β-dimethyl-aminoethyl-benzhydryl ether (Benadryl), N,N-dimethyl-N′-(2-pyridyl)-N′-(2-thenyl)-ethylenediamine (Histadyl), N′-p-methoxybenzyl-N′-(2-pyridyl)-ethylenediamine (Neoantergan), N,N-dimethyl-N′-(p-methoxybenzyl)-N′-(2-pyrimidyl)-ethylenediamine (Neohetramine), and 2-methyl-9-phenyl-1,2,3,4-tetrahydro-9H-Indeno [2, 1-c] pyridine (Thephorin). γ-Phenyl-γ-(2-pyridyl)-N,N-dimethylpropylamine (Trimeton) was highly erratic in its behavior. In general, the higher doses gave a degree of inhibition akin to that exhibited by the other antihistaminics, but the effects of the lower doses oscillated between inhibition and stimulation, a phenomenon never observed with the other drugs. This was particularly interesting because Trimeton has been said to lack local anesthetic action (6). More recently Tislow has reported that it does possess some local anesthetic action (7) but that in this respect it is much weaker than the other antihistaminic drugs. β-Diethyl-aminoethyl-p-aminobenzoate (Procaine) was well tolerated by the heart in much higher doses than any of the antihistaminics. It would thus appear that the effects of the antihistaminics on the heart cannot be due to their local anesthetic action; they may be an attribute of their specific antihistaminic action. If this latter hypothesis could be proved, the effects observed might be considered indirect evidence of a function of histamine in cardiac metabolism.

5. The various hearts exhibited both quantitative and qualitative differences in their responses to acetylcholine, carbaminoylcholine chloride (Doryl), and acetyl-β-methylcholine chloride (Mecholyl); but for the three drugs these differences were strictly parallel. These results emphasize the similarity in the mechanism of action of these parasympathomimetic drugs and also point to considerable differences in the parasympathetic architecture in these five species.

6. 2-Naphthyl-(1′)-methyl-imidazoline hydrochloride (Privine) inhibited all hearts, presumably by coronary constriction, which is additional indirect support for the contention that it acts very close to the final effector responses of the vascular muscle. The twofold action of Privine on the isolated cat heart, inconstant coronary constriction and a similarly inconstant positive inotropic effect, has already been described in detail (8).

That atropine and diethylaminoethyl-diphenylacetate hydrochloride (Trasentine) exhibited strictly parallel inhibitory effects is consistent with the reported significance of acetylcholine in cardiac metabolism (9). Finally, a careful analysis of the effects of the drugs upon the five species of hearts in the light of their known clinical effects would suggest the feline heart is closer in its metabolism to the human heart than are the other four species studied [cf. 2-benzylimidazoline hydrochloride (Priscoline), aminophylline, papaverine, and the parasympathomimetics].

SUMMARY

1. With the Langendorff preparation, the effects of a series of common drugs have been studied upon the perfused hearts of five species: cat, guinea pig, hamster, rabbit, and rat.

2. The significance of the results for a mechanism of drug action has been discussed from two standpoints:

(*a*) Drugs acting alike on all five species either act very close to, or directly upon, the final effector mechanism, or else upon a link of an enzymatic chain of reactions that is phylogenetically so significant as to be shared alike by all five species.

(*b*) Drugs of similar action sharing a like pattern of qualitatively differing responses upon the five species presumably possess a like mechanism of action.

The authors thank the following concerns for generous supplies of the indicated antihistaminics: Eli Lilly and Co., Histadyl; Hoffmann-La Roche, Inc., Thephorin; Parke, Davis and Co., Benadryl; Pyridium Corp., Neohetramine; and Schering Corp., Trimeton.

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The Preparation of Radioactive Morphine and Related Alkaloids by Biosynthesis*,†

By B. J. McINTOSH‡, F. E. KELSEY, and E. M. K. GEILING

Papaver somniferum **has been grown in a sealed atmosphere exposed to the radioactive isotope C^{14} in the form of $C^{14}O_2$, and a light intensity of 200 foot-candles. Under the conditions described *P. somniferum* incorporates carbon 14 into the morphine molecule after twenty-one days' exposure in sufficient quantities for metabolic studies. Morphine containing C^{14} was obtained by adsorption on Permutit from a solution in 50 per cent alcohol and then eluted with a compound having an ionization constant greater than 7.4×10^{-7}.**

THE extreme sensitivity of the radioactive isotope tracer technique has made possible many experiments on the metabolism of drugs not hitherto possible because of the limitations of available chemical and biological methods. Minute doses of many drugs produce profound pharmacological effects in the animal organism. In these instances it is especially difficult to carry out experiments on the distribution of the drug in the tissues of the body, on the rate and form of excretion products and on the nature of the metabolic products which may be formed in the body. The introduction of a radioactive isotope such as carbon 14 into the molecule of the drug under investigation makes possible the analysis of tissues and excreta for both the unchanged drug and for its metabolic products after the administration of subtoxic doses well within the therapeutic range. Morphine is a good example of a drug in this class. The studies reported here were undertaken to prepare usable quantities of radioactive morphine in order to carry out experiments on its intermediary metabolism. These will be reported at a later date.

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EXPERIMENTAL

Preparation of Radioactive Plants.—Seeds of *Papaver somniferum* were sown on black soil. When the seedlings had attained a size of approximately 10 cm., they were transplanted and the plants bottled as described in an earlier paper (1). Carbon dioxide was injected into the enclosed atmosphere surrounding the plant from a gas-tight syringe in which the carbon dioxide had been evolved by adding an excess of lactic acid to a solution of sodium carbonate.

The carbon dioxide concentration in the atmosphere surrounding the growing plants was determined by taking a measured amount of gas from the atmosphere and precipitating the carbon dioxide in a saturated solution of barium hydroxide. The amount of radioactive carbon in the sample was used as a measure of the amount of carbon dioxide in the atmosphere. All measurements of radioactivity were made in the counter previously described (2).

The sealed plants were placed under two 100-watt daylight-type fluorescent lamps which gave a light intensity of 300 foot-candles at the top of the battery jars, and between 150 and 250 foot-candles at the topmost leaves of the seedling. In a twenty-four hour period the lights were on for sixteen hours. The moisture was removed from the plant's atmosphere by a method published by Kimura (3).

At room temperatures above 22.5° the plants died within forty-eight to ninety-six hours. When the temperature was reduced below 22° the plants survived for periods up to and including thirty-six days. By maintaining the temperature between 14–18° it was not necessary to remove the moisture from the atmosphere. Plants grown under the latter condi-

tions were still in a moderately good condition at the end of thirty-five days. The temperature inside the battery jar was between 4° and 6° higher than the room temperature.

The radioactive poppy plants were dried at room temperature and used as the starting material for obtaining the radioactive morphine. Tests with minute samples of the green leaves exposed to carbon 14 revealed a considerable amount of radioactivity. The dried poppy hay, tested in a similar manner, showed marked radioactivity indicating clearly that the plant had taken up the carbon 14.

Extraction Procedures.—There are many methods (4) for the isolation of morphine from opium and poppy plants, but those methods whereby small amounts of the alkaloid can be purified to a state where crystallization can take place are fewer in number (5–7). Fulton (6) purified morphine contained in 0.5 Gm. flower petals or a "single green leaf of no great size" to a degree where he could identify it. The commonly used methods for crystallizing morphine are from dilute methyl alcohol (8), amyl alcohol (9), anisole (10), or by the addition of dilute ammonia to weakly acidic solutions of morphine. Morphine in amounts of 10 mg. or less was not crystallized by these methods except in certain unpredictable instances when long slender needles would crystallize from dilute methyl alcohol. These crystals were pure. Ammonium chloride was used to crystallize small amounts of morphine from an alcohol-ether-water system (5, 7).

Morphine as found in the plant is the same as that found in opium (6). The initial extracts from poppy hay and from opium have been made with water (4, 11), alcohol (6), and dilute acid (7). With large amounts of raw material, sodium carbonate can be used to precipitate the alkaloids.

In our experiments loss or destruction of the morphine was minimized by reducing the number of liquid-liquid extractions and keeping the morphine in either acidic aqueous solutions or organic solvents, except momentarily when the procedure required a basic solution. Pyrogallol was used by Morgan (12) to prevent the oxidation of morphine in alkaline solutions. This antioxidant was tried but it interfered with the extraction procedure to such an extent that its use was discontinued. Concentration of both the aqueous and chloroform extracts of the poppy hay was carried out *in vacuo* and on the steam bath, but there was no detectable difference in the resultant yield of morphine.

One of the best general techniques for the purification of small quantities of many substances is that of chromatographic adsorption (13, 14).

In 1938, Oberst (15) used hydrated aluminum silicate (Permutit) (13, 16) to adsorb morphine from urine extracts. He found that 1 mg. morphine in water solution was completely adsorbed by 1 Gm. Permutit. Permutit will adsorb morphine from alcoholic solution of concentrations as great as 95% (13). Merz and Franck (17) adsorbed certain alkaloids on alumina (Al_2O_3) and used 70% ethanol to elute them. In the elution of morphine from Permutit, a substance which has an ionization constant greater than that of morphine, 7.4×10^{-7}, will act as an efficient eluant. With alumina the eluant is determined by more empirical methods, but is usually an organic solvent.

Both of these adsorbents were used by us in preliminary investigations with morphine sulfate and the free base. Permutit was thoroughly washed with water and 50% ethanol in water. At least 10 mg. morphine sulfate could be adsorbed from a 50% ethanol solution by 1.7 Gm. of Permutit suspended in a column 0.5 x 14 cm. Permutit also adsorbed morphine dissolved in chloroform. Morphine sulfate adsorbed on Permutit was not eluted by absolute methanol or chloroform.

Alumina which had been washed with water, ethanol, and chloroform and activated by heating to 130° adsorbed the free base from chloroform. Inactivated alumina (activated alumina washed with water and alcohol) also adsorbed morphine. The disadvantage of this adsorbent was due to the firmness with which it adsorbed the morphine. Small volumes (10–50 ml.) of ethanol in chloroform, 95% ethanol, and 70% ethanol in water eluted small fractions of the morphine.

Codeine and thebaine are readily adsorbed by Permutit from either water or 50% ethanol solutions, but papaverine and narcotine are eluted with 50% ethanol. These properties were used to separate the phenanthrene derivatives from the benzylisoquinoline derivatives. The 50% ethanol filtrate also contained most of the pigment and other plant products which are extracted.

After a considerable amount of preliminary work with the above-mentioned techniques we decided upon the following procedure for the extraction of the morphine from our radioactive plants: two to four grams of dried poppy hay were placed in a Waring blendor with 100 ml. of 50% ethanol in water. After approximately ten minutes of blending the solution reached a temperature of 50°. The blending was continued for twenty minutes and the extraction repeated twice with fresh 50% ethanol. The combined extracts were filtered first through Whatman No. 40 filter paper and then through a sintered glass filter. The filtered extracts were either reduced in volume in some experiments or added directly to the column in others. If the volume is reduced a precipitate is formed which consists of the alcohol soluble, water-insoluble substances. A much larger adsorption column (1 x 50 cm.) was used in adsorbing leaf extracts than in the adsorption of pure morphine sulfate. Forty grams of Permutit in 50% ethanol was suspended in the column. This larger amount of Permutit prevents small amounts of salts, or perhaps other substances extracted from the leaves, from eluting the morphine.

Two hundred to 1000 ml. of 50% ethanol in water was used to elute completely the benzylisoquinoline derivatives and most of the coloring matter from the column. Water filtrates removed some more impurities. An ultraviolet absorption spectrum was made on the filtrates. If the eluate gave a density reading at 284 mμ greater than 0.100, 100 ml. more of the solvent was used. Ten-milliliter portions of 10% sodium chloride in water were then passed through the column and each 10-ml. filtrate analyzed for the phenanthrene derivatives by the use of the Beckman Spectrophotometer. With the quantity of Permutit used, the phenanthrene derivatives first appeared in the second filtrate. The third filtrate contained the highest concentration, while the fourth filtrate decreased to a value approxi-

mately half that of the third. The fifth and sixth filtrates did not give characteristic phenanthrene curves.

Codeine and thebaine were separated from morphine by making the sodium chloride filtrates basic with sodium hydroxide and extracting the aqueous layer four times with equal volumes of benzene. A grayish yellow precipitate was formed upon the addition of base, but no morphine could be detected in it.

Hydrochloric acid was used to acidify the aqueous portion and ammonia then added until the solution was alkaline to litmus paper. Six equal volumes of chloroform, or 2:1 chloroform-isopropyl alcohol, were used to extract the morphine from the ammoniacal solution. During the time that the morphine was in the alkaline solutions, the procedure was rushed as much as possible. The chloroform prevented complete crystallization of the morphine. Less than 0.5 ml. chloroform was used to redissolve the morphine, or if larger volumes were needed the excess chloroform was evaporated.

In most instances petroleum ether was used to crystallize the amorphous morphine, but ethyl ether plus petroleum ether served equally well. The crystals, when dissolved in the minimal amount of dilute hydrochloric acid, gave the characteristic acid morphine ultraviolet absorption curve; gave the Prussian blue test for the phenolic hydroxyl group; and when injected gave the characteristic Straub mouse test for morphine and its esters. Dried crystals turned purple, then green when a drop or two of a solution of molybdic acid in concentrated sulfuric acid was placed on them (18). This latter test can be used as a means of differentiating many of the opium alkaloids. Pseudomorphine turns blue when acted on by this reagent. Medicinal opium samples subjected to this procedure have shown an 8% morphine content, or approximately 80% of the theoretical value (5).

RESULTS AND DISCUSSION

Tables I and II give a comparison of the relative amounts of radioactivity incorporated into the morphine molecule from two groups of plants which were exposed to $C^{14}O_2$ for different lengths of time. Those plants, from which Table I was composed, were exposed to 300 μc C^{14} for eight days. At the end of this eight-day exposure, the plants were harvested but many plants had already died or begun to rot. Pods, both in a semiripe and a ripe condition, were found on almost all of the plants. One gram of the 4.1 Gm. total weight of hay was used for the extraction procedure. Even though the sodium chloride eluates also contained codeine and thebaine, their concentrations were probably so small that they did not add materially to the total spec-

TABLE I.—RADIOACTIVITY IN FILTRATES OF PERMUTIT COLUMN

Filtrate Number (1)	Description (2)	Total Vol. of Filtrate, (Ml.) (3)	C. p. m./Ml. Filtrate[a] (4)	Density Reading at 284 mμ (5)
1	50% alcohol	40.0	11,332	...
2	50% alcohol	41.0	558	...
3	50% alcohol	40.0	965	...
4	50% alcohol	40.0	662	...
5	50% alcohol	46.5	516	...
6	Water	55.0	779	...
7	Water	50.0	452	...
8	Water	50.0	172	...
9	Water	32.0	132	...
10	10% NaCl	13.0	156	1.07
11	10% NaCl	10.0	490	1.11[b]
12	10% NaCl	10.0	349	∞[b]
13	10% NaCl	10.0	165	1.68[b]
14	10% NaCl	10.0	109	0.497
15	10% NaCl	10.0	109	0.311
16	10% NaCl	12.0	55	...
17	Dilute NH_3	81.5	566	...

[a] The filtrate was evaporated to dryness on a copper disk and counted.
[b] Contains morphine.

TABLE II.—RADIOACTIVITY IN FILTRATES OF PERMUTIT COLUMN

Filtrate Number (1)	Description of Filtrate (2)	Vol. of Filtrate, Ml. (3)	C. p. m./Ml. Filtrate[a] (4)	Density Reading at 284 mμ (Sample Diluted 1:4) (5)
1	10% NaCl	10.0	13,936	0.042
2	10% NaCl	9.7	20,015	0.045
3	10% NaCl	9.8	210,345	1.02[b]
4	10% NaCl	9.9	152,665	0.634[b]
5	10% NaCl	9.6	67,005	0.288[b]
6	10% NaCl	10.0	35,525	0.158
7	10% NaCl	26.5	11,487	...
8	10% NaCl	27.0	5,935	...

[a] The filtrate was evaporated to dryness on a copper disk and counted.
[b] Contains morphine.

trophotometric reading (18). No ultraviolet analysis was made of the last water filtrate since two previous experiments, using the same amount of hay, showed that the last 50-ml. water filtrate gave a density reading at 284 mμ of less than 0.100. Comparison of columns 4 and 5, Table I, shows that as the concentration of morphine in the sodium chloride filtrates increased in filtrates 11 and 12, the amount of radioactivity in the two filtrates decreased. This fact would indicate that the exposure period had been too short for C^{14} to be incorporated into the morphine molecule.

As a control experiment radioactive digitalis leaves which were exposed to $C^{14}O_2$ for approximately thirty days were extracted three times with 95% ethanol and the extract added to a Permutit column (0.5 x 14 cm.). The first two 95% ethanol filtrates contained too high a radioactivity concentration to be counted. The third 95% ethanol filtrate contained 20,195 counts per minute/milliliter (c. p. m./ml.). The following 90% ethanol filtrate contained 34,224 c. p. m./ml., 80% ethanol contained 21,248 c. p. m./ml., 60% ethanol contained 11,072 c. p. m./ml., and 50% ethanol contained 4416 c. p. m./ml. One milliliter of the 25-ml. water filtrate which followed the 50% ethanol filtrate contained 4736 c. p. m./ml., and the next filtrate, 1% sodium chloride, contained 1344 c. p. m./ml. If the components of the two plants, *Digitalis purpurea* and *Papaver somniferum*, are approximately the same, with the exception of the glycosides and alkaloids, this experiment would indicate that the Permutit concentrates the phenanthrene derivatives without concentrating other substances, although some other substances are eluted with NaCl besides the phenanthrene group.

The plants referred to in Table II received a total of 1,125 μc over a period of twenty-one days. The total growing period, after the first carbon dioxide injection, was twenty-four days. The green plants contained 80% water by weight. Eighty-one per cent (2 Gm.) of the total dried plant material was used in the extraction. There is a marked contrast between the amount of radioactivity in the sodium chloride filtrates of Tables I and II. That the morphine (or at least the phenanthrene derivatives) contained C^{14} is shown by comparing columns 4 and 5. As the morphine concentration in the sodium chloride eluate increased and decreased (as determined spectrophotometrically) the c. p. m./ml. in each eluate also increased and decreased.

Crystallization of the morphine from chloroform with petroleum ether gave a total of 10.5 mg. morphine (0.52% of the hay). There were 84×10^3 c. p. m./mg. morphine[1] in the purified product, as opposed to 144×10^3 c. p. m./mg. crude morphine eluted in the sodium chloride filtrates 3, 4, and 5.

SUMMARY

Papaver somniferum can be grown in a sealed atmosphere for at least thirty-six days. Temperatures below 22° and above 14° are quite satisfactory for extended periods (thirty-six days of plant growth. At temperatures between 14–18° the high relative humidity does not seem to be deleterious, but at higher temperatures a part of the moisture must be removed from the sealed atmosphere. Although light intensities which approached 300 foot-candles were used, a light source with greater candle power would probably be desirable.

P. somniferum incorporates carbon 14 into the morphine molecule after twenty-one days' exposure to $C^{14}O_2$. A light intensity of approximately 200 foot-candles is necessary for growing periods which are of a sufficient length of time to allow radioactive morphine to be synthesized. Plants grown in a $C^{14}O_2$ atmosphere for less than eight days produced small amounts, or no radioactive morphine.

Morphine which contains C^{14} can be obtained from the plant by adsorbing morphine on Permutit from a solution of 50 per cent ethanol in water and eluting it with a compound having an ionization constant greater than 7.4×10^{-7}. Morphine is not eluted from Permutit by petroleum ether, ethyl ether, carbon tetrachloride, acetone, or absolute methanol. Morphine is adsorbed by Permutit from chloroform. Papaverine is adsorbed by Permutit from aqueous solutions and eluted with 50 per cent ethanol in water. Narcotine is adsorbed from an ether solution and eluted with ethanol. Codeine and thebaine are adsorbed by Permutit from aqueous or 50 per cent ethanol solutions and eluted with sodium chloride in water.

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[1] The morphine base was dissolved in dilute hydrochloric acid and an aliquot of the solution was evaporated on a copper disk and counted.

The Pharmacology of Lunarine, the Alkaloid of *Lunaria biennis*[*,†]

By F. G. HENDERSON and K. K. CHEN[‡]

General pharmacologic studies of this alkaloid are presented including effects on the cardiovascular system, smooth muscle, carbohydrate metabolism, and glandular secretions. The general reaction of rabbits was recorded and intravenous toxicity studies were carried out in albino mice.

LUNARIA BIENNIS is a widespread crucifer which belongs to the group of *Siliculosae dehiscentes*, subgroup *pleurohiziae*. It is frequently cultivated in Europe as a decorative plant. In 1909 Hairs (2) isolated an alkaloid from the seeds of *L. biennis*. Almost at this same time Reeb (3) prepared a similar alkaloid which he named lunarine, and described its paralyzing effect on the central nervous system of rabbits and frogs. Chemical investigations on lunarine were carried out by Hansen (4). He proposed the empirical formula $C_{25}H_{33}N_3O_5$. Independently, Steinegger and Reichstein (5) of Basel, Switzerland, studied the same alkaloid which they isolated in crystalline form from the seeds of *L. biennis*. Their combustion analysis conformed to the empirical formula $C_{19}H_{29}N_3O_4$.

The hydrochloride is colorless and has a melting point of 227–231° (corr.). It is soluble in water and optically active. Steinegger and Reichstein (5) made the following observations in regard to the chemical structure: (*a*) there are two nitrogen atoms, but only one of them possesses basic properties; (*b*) it is not a primary aromatic amine; (*c*) it contains neither methoxyl nor methylamide groups; and (*d*) it does not contain easily hydrolyzable ester compounds.

Our studies of this alkaloid were made possible by the generous sample of lunarine HCl supplied to us by Professor T. Reichstein, University of Basel. The purpose of this paper is to present the pharmacologic data thus far accumulated.

EXPERIMENTAL

Action on the Cardiovascular System.—In the barbitalized cat and dog, lunarine HCl in the dose of 5 mg./Kg. by vein caused a marked fall in arterial blood pressure accompanied by an increase in rate and a decrease in depth of respiration. As shown in Fig. 1, the drop in blood pressure was very rapid and persisted for a period of twenty-five to thirty minutes. In this particular experiment a dose of 5 mg./Kg. caused death of the dog. However this animal had previously received various doses of lunarine HCl. Kidney volume decreased as the blood pressure fell, indicating that there was no dilation of the renal vessels. Further studies on the isolated perfused kidney also showed evidence of constriction of the renal vascular system. Lunarine HCl in a concentration of 1:5000 decreased the flow of the perfusate through the kidney from 39 to 32 drops per minute.

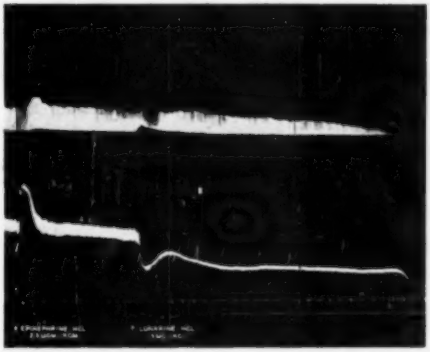

Fig. 1.—Arterial blood pressure, respiration, and kidney volume. The tracings from above downward are kidney volume, respiration, blood pressure, and time in minutes. The dog was anesthetized with "Amytal Sodium" (Amobarbital Sodium, Lilly) and all injections were given by the intravenous route.

Studies on the peripheral circulation were carried out by observing changes in the volume of the hind leg of the dog (Fig. 2). The increase in the volume of the leg following intra-arterial injection of 20 mg. of lunarine HCl was of the same magnitude as that following intra-arterial injection of 25 μg. of histamine, but it was of longer duration. This would indicate that peripheral vasodilation is one of the factors responsible for the fall in blood pressure.

We were unable to show any direct effect on the heart *in situ* in dogs. Serial electrocardiograms made before and during the fall in blood pressure following lunarine HCl by vein in dogs failed to show any changes.

Perfusion studies on the rabbit and frog heart indicated some direct action of lunarine on the heart in concentrated solutions. One of 1:2500

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† Presented in part at the fall meeting of the American Society for Pharmacology and Experimental Therapeutics, Indianapolis, 1949 (1).
‡ The authors are deeply indebted to C. E. Powell and H. E. Roeder for their invaluable assistance in completing this study.

Fig. 2.—The effect of lunarine HCl on leg volume. A mongrel dog was anesthetized with "Amytal Sodium" and leg volume recorded of left hind leg. Injections were made into the femoral artery. Tracings from above downward are leg volume, blood pressure, and time in minutes.

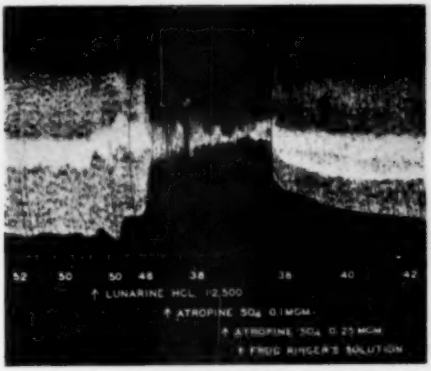

Fig. 4.—Perfused frog heart. The frog heart was perfused by the method of Howell and Cooke. The numbers in the legend indicate heart rate.

decreased the rate and amplitude of the isolated rabbit heart (Fig. 3), perfused by the Langendorff (6) method. The ventricular rate dropped from 104 to 68 beats per minute. This was followed by a return to normal.

In the frog heart, perfused according to the method of Howell and Cooke (7), a concentration of 1:2500 decreased the amplitude of contractions but had very little effect on the rate (Fig. 4). The addition of atropine in quantities of 0.1 mg. and 0.25 mg. failed to change this response. In one frog the same concentration produced a complete auriculo-ventricular block with periodic ventricular contractions coming through.

Fig. 3.—Isolated rabbit heart. The isolated rabbit heart was perfused by the Langendorff method. The numbers in the legend indicate heart rate.

Action on Smooth Muscle.—The effect of lunarine HCl on intestinal motility was studied in dogs. Records were made of duodenal motility by placing a balloon in the second part of the duodenum of anesthetized dogs. Lunarine HCl in a dose of 5 mg./Kg. caused a marked ncrease in duodenal activity (Fig. 5). This stimulation could be prevented in the anesthetized dog by atropine sulfate in a total dose of 1 mg. (0.122 mg./Kg.).

Intestinal motility studies were also made on nonanesthetized dogs with Thiry fistulas. When lunarine HCl in the dose of 5 mg./Kg. was given by vein it increased the motility of both ileac and jejunal portions of the small intestine. Figure 6 shows the pattern of stimulation produced in a dog with an ileac fistula. The tone is markedly increased, but there is little change in segmental movements. Following atropine, in two successive doses of 0.1 mg./Kg. each, the stimulating effect of lunarine could still be elicited.

Contrary to the *in vivo* findings lunarine did not alter the motility of the isolated gut. Concentrations of 1:10,000 had no effect on the isolated rabbit ileum.

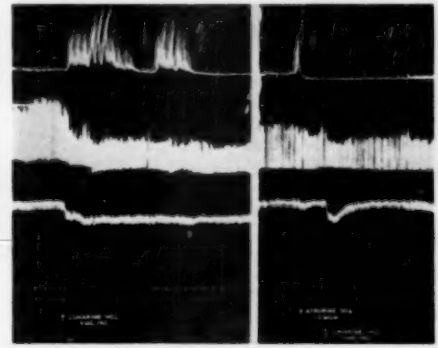

Fig. 5.—Effect of lunarine HCl on intestinal motility in the anesthetized dog. This dog was anesthetized with "Amytal Sodium" and tracings made of duodenal motility, respiration, and blood pressure. All injections were made by vein.

From the above results we thought this alkaloid might have some anticholinesterase activity, but studies in the Warburg apparatus on rat blood and brain *brei* according to the method of Augustinsson (8) gave rise to negative results.

Lunarine HCl did not affect the nonpregnant cat uterus or the isolated rabbit or guinea-pig uterus. It did inhibit the spontaneous contractions of the isolated rat uterus as shown in Fig. 7.

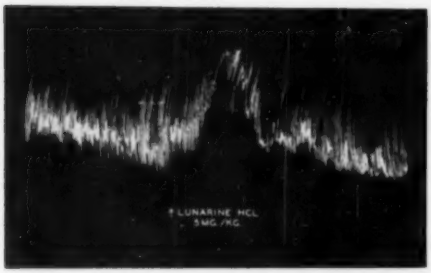

Fig. 6.—Action on intestinal motility in the non-anesthetized dog. This tracing shows intestinal motility recorded in a trained dog with a Thiry fistula of the lower portion of the ileum. Lunarine was given by the intravenous route.

A two per cent solution of lunarine HCl placed in the conjunctival sac of albino rabbit eyes produced a slight myosis. This was of short duration, and could hardly be accepted as evidence of a cholinergic substance.

Carbohydrate Metabolism.—Lunarine HCl was given in the dose of 20 mg./Kg. by vein to fasted albino rabbits. Blood sugar determinations were made at periodic intervals following injection. This dose produced no significant changes in blood sugar.

Fig. 7.—Effect on the isolated rat uterus.

Secretory Effect.—Following cannulation of the pancreatic and submaxillary ducts of an anesthetized dog, lunarine HCl was given by vein. A dose of 5 mg./Kg. did not alter the flow of secretion from either gland. However, following pilocarpine the same dose decreased the salivary flow. This may be attributed to the fall in blood pressure following lunarine.

Effect on the Nervous System.—The effect of lunarine on the central nervous system was studied in albino rabbits. The alkaloid was given by vein and the general reaction of the rabbits observed. Doses of 20 and 30 mg./Kg. caused a decrease in respiratory rate and a labored type of respiration. The animals returned to normal in a period of six to eight minutes. A dose of 50 mg./Kg. produced head drop, constriction of the pupils, complete relaxation of the extremities, and a decrease in respiratory rate from 192 to 40 per minute. Six minutes after injection the animal righted itself and was able to hold its head up, and in eleven minutes it returned to normal. A dose of 60 mg./Kg. gave the same picture including convulsive twitches of the extremities; it required nineteen minutes for the animal to return to normal. A dose of 70 mg./Kg. caused almost immediate death following respiratory failure. The effect of lunarine HCl on the central nervous system of rabbits agrees very well with the description given by Reeb (3).

Because of the head drop produced in rabbits, the alkaloid was examined for curariform action. An anesthetized rat was prepared to record contractions of the gastrocnemius muscle following stimulation of the sciatic nerve. Lunarine HCl was given by vein in doses ranging from 5–40 mg./Kg. There was no evidence of block at the myoneural junction. Thus, the head drop and muscular relaxation are probably due to the effect on the central nervous system.

Toxicity.—The acute toxicity of lunarine HCl was determined in albino mice by intravenous injection. All deaths were preceded by violent clonic convulsions. In smaller doses there appeared to be some muscle weakness, but the mice did not show the paralysis as seen in rabbits. Deaths appeared to be due to respiratory failure. The median lethal dose (LD_{50}) as determined in 45 animals was 62.3 mg./Kg.

DISCUSSION

From the above data the exact nature of the pharmacologic action of this compound cannot be classified. Interpretation of several effects may be offered:

(*a*) The fall in blood pressure is due primarily to dilatation of peripheral vessels. The splanchnic vessels show no evidence of dilatation. It required a very high concentration to alter the activity of either the mammalian or amphibian heart.

(*b*) This alkaloid *in vivo* stimulated movements of the small intestine. This was not true with isolated intestinal strips. Thus, this phenomenon is not due to direct action on intestinal musculature.

(*c*) This compound showed no anticholinesterase activity.

(*d*) The lack of secretory effect, the minimal myosis, the absence of stimulating effect on the isolated uteri, in fact inhibition of the rat uterus, are against the classification of this alkaloid as a parasympathomimetic.

(*e*) The head drop produced in rabbits is due to the depressant effects on the central nervous system.

SUMMARY

1. Lunarine HCl in a dose of 5 mg./Kg. by vein lowered blood pressure, increased respiratory rate, and decreased kidney volume in the anesthetized dog. Intra-arterial injections into the hind leg of the dog decreased the leg volume.

2. Perfusion of the isolated kidney with this alkaloid in a concentration of 1:5000 showed a tendency to vasoconstriction.

3. There were no changes in the dog heart, *in vivo*, as shown by ECG recordings made follow-

ing the injection of lunarine. A concentration of 1:2500 inhibited the perfused frog and rabbit heart.

4. This alkaloid stimulated the intestinal motility of both the anesthetized and nonanesthetized dog. A concentration of 1:10,000 did not alter the spontaneous contractions of the isolated rabbit intestine.

5. Lunarine did not stimulate secretions of the pancreas or submaxillary glands.

6. In rabbits it produced head drop, depressed respiration, and caused constriction of the pupils.

7. The median lethal dose (LD_{50}) as determined in albino mice by intravenous injection was 62.3 mg./Kg.

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Assay of Official Calcium Products by the Schwarzenbach Method*

By ALBERT M. MATTOCKS and HECTOR R. HERNANDEZ

The Schwarzenbach method for determination of calcium and magnesium in water has been applied to the analysis of several official calcium salts. The results by this method are accurate and the procedure is much more rapid and convenient than the methods now official.

RECENTLY the Schwarzenbach method for the determination of calcium and magnesium in water as a test for hardness has received much attention (1–4), and a reliable procedure has been developed. This method consists of titrating the calcium contained in solution with disodium ethylenediamine tetraacetic acid (ETA), in the presence of a small amount of magnesium. Eriochrome Black-T is used as an indicator and this substance at *p*H 10 is clear blue in color but changes to a wine-red in the presence of free magnesium ions, the change being sharp and distinct. The reagent, ETA, reacts preferentially with the calcium; thus when enough calcium is present to utilize all of the reagent, the small amount of magnesium present will be left free to react with the indicator.

The convenience of the above method led the authors to investigate its application to pharmaceutical products containing calcium. For initial investigations the assays of the calcium salts of the National Formulary were studied, and subsequent work will be concerned with applying this method to the assay of the calcium salts of the U. S. Pharmacopeia and the magnesium salts of both compendia.

* Received July 19, 1950, from the Laboratory of the AMERICAN PHARMACEUTICAL ASSOCIATION.

EXPERIMENTAL

In preliminary work calcium salts were dissolved in water, or in dilute HCl when required, *p*H 10 buffer and 4–5 drops of indicator were added and solutions were titrated with ETA containing small amounts of magnesium chloride. End points were sharp but variable results were obtained. At this point it was noted that a small amount of precipitate was present in the solution at the end of titration, and this was suspected to be calcium hydroxide which had come out of the basic solution before sufficient ETA had been added. It was found, therefore, that a much more reliable procedure consisted of adding an excess ETA before making the solution alkaline, inasmuch as the ETA would complex the calcium and prevent its precipitation from alkaline solution. The excess ETA could then be titrated with a standardized magnesium chloride solution. The procedure in detail is as follows.

ETA Reagent.—Dissolve about 10 Gm. of disodium ethylenediamine tetraacetic acid in sufficient water to make 1 L. (There is some controversy over whether this solution would be stored in soft or hard glass vessels, but the authors found their solutions to be reasonably stable in Pyrex bottles.)

Magnesium Chloride Solution.—Dissolve 7.5 Gm. of reagent magnesium chloride in sufficient water to make 1 L.

Buffer Solution.—Dissolve 67.5 Gm. of ammonium chloride and 570 cc. of concentrated ammonium hydroxide in enough water to make 1 L.

Indicator Solution.—Dissolve 5 Gm. of ManVer indicator powder (Hach Chemical Company, Ames, Iowa), consisting of 1 part eriochrome Black-T and 9 parts of hydroxylamine hydrochloride, in sufficient methanol to make 100 cc.

Primary Standard.—Dissolve 1.000 Gm. reagent calcium carbonate in dilute hydrochloric acid and dilute to 1 L. It is difficult to obtain a reagent calcium carbonate that contains less than 0.5% magnesium and other alkalies, and it may be necessary to correct the strength of this solution by assaying it by the oxalate method.

Standardization of Magnesium Chloride Solution.—Transfer 50 cc. of ETA solution to a 250-cc. flask, add four drops indicator solution and 5 cc. buffer, and titrate with magnesium chloride solution. Calculate the ratio $cc._{ETA}/cc._{MgCl_2}$.

Standardization of ETA Solution.—Fifty cubic centimeters of the calcium carbonate primary standard solution is pipetted into a 250-cc. flask, 50 cc. of water is added, about 40 cc. of ETA solution, accurately measured, and four drops of indicator are then added to the flask, and the solution is titrated with magnesium chloride. The strength of the ETA solution is then calculated as the molarity in equivalents of calcium by the following expression:

$$\text{Molarity of ETA} = \frac{\text{Gm. } CaCO_3 \text{ used} \times 1000}{[cc._{ETA} - (cc._{MgCl_2} \times R)] \times \text{molecular weight } CaCO_3}$$

Titration of Calcium Salts.—An amount of calcium salt equivalent to about 40–50 mg. of calcium, is weighed into a 250-cc. flask, and if soluble the salt is dissolved in water, if not, it is dissolved in a small amount of dilute hydrochloric acid. Fifty cubic centimeters of water, 40–50 cc. of ETA solution, accurately measured, and six drops of indicator are then added, and solution is titrated with magnesium chloride solution. The per cent purity is calculated by the following equation:

$$\% = \frac{[cc._{ETA} - (cc._{MgCl_2} \times R)] \times \text{molarity ETA} \times \frac{\text{molecular weight}}{\text{No. Ca per molecule}} \times 100}{1000 \times \text{weight sample}}$$

Results obtained by the method described above and by the official method for the several calcium salts of the National Formulary are shown in Table I.

TABLE I

Substance	Purity—Per Cent Official Method	Purity—Per Cent ETA Method
Calcium bromide[a]	75.0	75.5
	74.4	74.7
Calcium levulinate	100.8	100.4
	100.8	99.7
	99.8	100.1
Calcium hypophosphite[b]	...	100.5
	...	100.7
	...	100.7
Calcium phosphate[b]	...	97.1
	...	96.8
	...	96.7
Calcium glycerophosphate	87.2	88.6
	87.2	88.2
	87.2	88.4
Lime	89.7	93.8
	90.0	92.9
		93.5

[a] Hygroscopicity of the salt caused slight errors in weighing which account for deviations.

[b] The low sensitivity of the official method, coupled with the fact that it does not measure the calcium ion, made it seem inadvisable to attempt a comparison.

DISCUSSION AND CONCLUSIONS

The official oxalate method for calcium requires considerable expenditure of time. There is a two-hour period of digestion in the method and the precipitate of calcium oxalate has to be filtered out, washed, and dissolved. This series of manipulations is tedious and likely to cause errors unless considerable care is exercised. Due to small losses incurred in filtering and washing the precipitate there is a tendency for results to be low. The ETA method requires not more than fifteen minutes for the assay of a pair of samples including their weighing, and a high precision is obtained. This represents a considerable improvement over the official oxalate method. Also it has been found in some cases, i.e., calcium hypophosphite, tribasic calcium phosphate, that the ETA method is satisfactory, whereas the oxalate method cannot be applied.

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The Potentiometric Titration of the Sulfa Drugs*

By JOSEPH P. LaROCCA and KENNETH L. WATERS

The potentiometric titration of the sulfa drugs is reported. The official assay is modified in that the end point of the titration is determined potentiometrically and the use of ice has been eliminated. Comparison with the official assay is favorable since the method is rapid, convenient, and consistent results are obtained. The method does not permit the human error variation in determining the sometimes indefinite blue starch-iodide end point Indications are that the modified procedure could have extensive use in pharmaceutical control.

THE general utility of potentiometric methods in the pharmaceutical control laboratories has been adequately demonstrated by many recent papers. Excellent reviews by Furman (1, 2) cover the major advances in the field of potentiometric titration. Herd (3) has indicated the possible value of potentiometric titration in the estimation of alkaloids. The iodometric-potentiometric titration of ascorbic acid has also been reported (4). Waters, *et al.* (5) have reported the potentiometric titration of the barbiturates, their sodium salts, and other organic salts. Mattocks and Voshall (6) have developed a titrimetric procedure whereby phenobarbital elixir could be titrated directly with silver nitrate, using a silver, silver-silver chloride electrode system.

When the solution of an aromatic amine is diazotized, free nitrous acid is formed at the completion of the reaction. A solution of nitrous acid in contact with a platinum electrode gives a definite potential. Muller and Dachselt (7) studied the titration of various aromatic amines and found that there is a jump in potential at the equivalence point of the titration of the acid solution of the aromatic amine with sodium nitrite. The official method for the quantitative determination of the sulfa drugs is dependent on the quantitative conversion of the aromatic amino group into a diazo group by the action of nitrous acid. The end point of the diazotization is indicated by the first slight excess of nitrous acid that is stable for at least one minute. This end point is detected by means of a spot test using potassium iodide-starch as an external indicator. The course of the reaction is followed by spot testing at intervals. These spot tests are made by withdrawing a drop of the solution and streaking potassium iodide-starch paper. The equivalence point is reached when, upon testing, a distinct blue color is obtained immediately and is reproducible after one minute. Difficulty is experienced in establishing the exact end point by this visual method. To eliminate this human factor, a method of determining this end point by potentiometric titration has been utilized.

Nicholas (8) has reported the electrometric titration of sulfanilamide. This worker stated that the end point obtained by the electrometric method did not agree with the one obtained by the official British Pharmacopeia method. The article gave no analysis figures nor was any attempt made to explain the discrepancies other than criticizing the method employed by the British Pharmacopeia in the standardization of the sodium nitrite solution.

In view of the previous work cited above, it was thought that a satisfactory method utilizing potentiometric titration could be adapted to the quantitative determination of the sulfa drugs. This method was investigated and found to give excellent results in the assay of all the sulfonamides official in the U. S. P. XIII (9). The method developed is rapid, convenient, and accurate. Results obtained in this investigation did not indicate the necessity of using ice in the titration mixture. This fact is further substantiated by the work of Singh and Ahmed (10) which showed that in the potentiometric titration of aromatic amines it was not necessary to add ice to the titration mixture since the error due to side reactions was found to be only 3 per cent at 60°; 0.5 per cent at 30°; and negligible at 20°.

EXPERIMENTAL[1]

Potentiometric Titration of the Sulfa Drugs with Sodium Nitrite.—Both a Beckman Potentiometer equipped with outside electrodes and a Fisher Senior Titrimeter were employed in this investigation. A platinum electrode and a calomel electrode were used in connection with the potentiometers.

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[1] The authors wish to thank Sharp & Dohme, Inc., for supplying samples of Sulfasuxidine, sodium sulfadiazine, and sodium sulfamerazine; and Lederle Laboratories for supplying samples of sulfaguanidine and sulfamerazine used in this investigation.

TABLE I.—RESULTS OF POTENTIOMETRIC ASSAY OF SULFONAMIDES

	Powder				Tablets[a]			
Sulfonamide	U. S. P. Assay	Av.	Potentiometric Assay	Av.	U. S. P. Assay	Av.	Potentiometric Assay	Av.
Sulfanilamide	...	...	...	...	93.0		94.9	
	...		...		93.4		95.2	
	...		...		92.5	92.9	95.2	95.1
Sulfathiazole	99.2		99.9		100.2		98.7	
	99.2		99.8		100.6		98.7	
	99.3	99.2	99.8	99.8	99.8	100.2	98.7	98.7
Sulfaguanidine	99.4		100.1		94.5		96.8	
	100.1		100.1		96.7		99.2	
	99.1	99.6	99.7	99.9	95.8	95.7	98.0	98.0
	...		99.7					
Sulfamerazine	99.5		99.6		95.5		99.0	
	99.6		99.6		96.8		98.7	
	99.8	99.6	99.6	99.6	96.7	96.3	98.7	98.8
Sulfadiazine	95.2		...					
Sample I	94.3		94.7					
	94.3		94.7					
Sample II	99.2		99.9		95.8		98.9	
	99.4	94.6	99.9	94.7	98.3		98.9	98.9
	99.3	99.3	99.9	99.9	97.5		...	
Sulfasuxidine	100.0		100.1		96.1	97.3	99.3	
	99.4	99.7	100.1	100.1	96.1	96.1	98.9	
	...		100.1		...		98.9	99.0
Sulfamerazine Sodium	99.1		100.0					
	99.2		99.7	99.9				
	99.1	99.1	...					
Sulfadiazine Sodium	99.9		100.1					
	99.5	99.7	99.7	99.9				
Sulfathiazole Sodium	100.8		99.8					
	100.4		99.4	99.6				
	100.2	100.5	...					

[a] Percentage expressed in terms of labeled amount according to U. S. P. XIII.

The titrant was added in 0.1-ml. portions and the end point was determined by means of differential curves (Δ_p/Δ ml. vs. ml.). For all practical purposes, it was found that the volume of titrant added at the point of maximum rise in potential was the equivalent volume, thereby eliminating the necessity of drawing the differential curves. The following procedure was employed in the assay of the sulfonamides: the samples were prepared for titration according to the procedure given in the U. S. P. XIII for the various sulfa drugs. The solution was then placed on the titrimeter, which had been fitted with platinum and calomel electrodes. No attempt was made to titrate to a definite potential, but rather to titrate to a maximum rise in potential. No ice was added to the solution and the titrant was added in 5-ml. portions up to within 1 ml. of the calculated equivalence point. The titrant was then added in 0.1-ml. portions until a maximum rise in potential was obtained. Appreciable time lags were experienced in the first additions of sodium nitrite; however, as the equivalence point was approached, especially after the 0.1-ml. additions, the reaction was complete within one minute. All samples were assayed both potentiometrically and by the official method. The sodium nitrite solution was standardized using pure sulfanilamide as the reference standard. The sodium nitrite was standardized by both methods and the molarity obtained by each was used in the calculations of the assays by that method. This procedure was necessary since the molarity as determined by the individual methods differed significantly.

Eight sulfonamide powders and six sulfonamide tablets were assayed. The results are shown in Table I. In general, the results obtained by the potentiometric method were slightly higher and more precise than those obtained by the U. S. P. method. The data would indicate that a higher degree of accuracy is attained in the potentiometric assay than in the official method.

SUMMARY

1. A potentiometric method was utilized for the assay of various sulfonamide powders and tablets with 0.1 *M* sodium nitrite, using platinum, calomel electrodes.

2. This procedure eliminates the necessity of ice in the titration mixture and removes the human variation in determining the end point.

3. This procedure was found to give very accurate results in the assay of sulfathiazole, sulfaguanidine, sulfamerazine, Sulfasuxidine, sulfamerazine sodium, sulfadiazine sodium, and sulfathiazole sodium powders. Excellent results were also obtained in the assay of sulfanilamide, sulfathiazole, sulfaguanidine, sulfadiazine, sulfamerazine, and Sulfasuxidine tablets.

4. Indications are that the modified procedure could have extensive use in pharmaceutical control.

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Rapid Acidimetric Determination of Alum in Alum-Precipitated Biologicals*

By MORRIS B. JACOBS†,‡

A method for the rapid acidimetric estimation of alum in alum-precipitated toxoids is presented. It is based on the solution of the alum-precipitated biological in a dilute acid-citrate solution, the buffering of this mixture with a tartrate buffer to permit rapid reaction, the neutralization of the test mixture with barium hydroxide, sequestration of the aluminum with potassium fluoride with consequent liberation of three moles of potassium hydroxide for every mole of aluminum, and finally estimation of the liberated alkali acidimetrically. This method is less subject to error, particularly error attributable to iron, than gravimetric methods now in use and has adequate precision.

THE National Institutes of Health have set certain standards (1, 2) for the production of alum-precipitated toxoids and other biologicals. This standard with respect to alum-precipitated diphtheria toxoid states: "3.1. The finished toxoid shall contain not more than 15 mg. of alum in any individual human injection as determined by analysis of the finished product. In lieu of such analysis the amount shall not be more than a calculated 20 mg. based on the assumption that the total amount added is recovered in the finished product." The requirement for alum-precipitated tetanus toxoid is the same. The method of analysis is not specified but it is customary for manufacturers to file the method they use with the National Institutes of Health.

One of the methods used for the estimation of alum in such biologicals is the gravimetric method described by Berger (3). This method is long and tedious and has an inherent error in that iron is determined as aluminum in this proximate analysis. A colorimetric method involving the formation of an aluminum lake with alizarin red (4) has also been employed.

The proposed method is rapid, more accurate than the gravimetric method and the alizarin red method, and has adequate precision for the purpose for which it is to be used.

It is based on the solution of the alum-precipitated biological in a dilute acid-citrate solution, the buffering of the mixture with a tartrate buffer, the neutralization of the test mixture with barium hydroxide, sequestration of the aluminum by use of potassium fluoride with consequent liberation of three moles of alkali hydroxide for every mole of aluminum,

$$Al^{+++} + 3OH^- \rightarrow Al(OH)_3$$

$$Al(OH)_3 + 6KF \rightarrow AlF_3 \cdot 3KF + 3KOH$$

and finally estimation of the liberated alkali acidimetrically.

This method is then a variation of the methods of Viebock and Brecher (5) and of Snyder (6) based on prior work of Craig (7), Scott (8), Travers (9), and Malaprade (10).

METHOD

Reagents

Potassium Fluoride, 30% Solution.—Dissolve 300 Gm. of reagent grade potassium fluoride in water and make up to 1 liter with water. Store in a wax-lined bottle.

Potassium Sodium Tartrate, 30% Solution.—Dissolve 300 Gm. of reagent grade Rochelle salt in water and make up to 1 liter with water.

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‡ I wish to thank Mr. David L. Shepard for his assistance in this investigation.

Barium Hydroxide, Saturated Solution.—Heat 60 Gm. of barium hydroxide, $BaOH \cdot 6H_2O$, or 30 Gm. of BaOH in 1 liter of water to boiling, allow to cool, and filter into a stock bottle.

Hydrochloric Acid, 0.3 *N*.—Prepare an approximately 0.3 *N* hydrochloric acid solution in the conventional manner but standardize it in terms of aluminum as described below.

Weigh out accurately as closely as possible 26.95 Gm. of pure aluminum wire and dissolve in 200 ml. of 1 *N* carbonate-free sodium hydroxide solution. Make the solution just acid with hydrochloric acid (1:1) and boil gently to expel carbon dioxide. Dilute to 1 liter. Standardize aliquots according to the procedure detailed in the method.

Hydrochloric Acid, 0.03 *N*.—Prepare a 0.03 *N* hydrochloric acid solution from the 0.3 *N* hydrochloric acid in the conventional manner. Standardize against the standard alum solution or in a manner similar to that given above.

Standard Alum Solution.—Weigh out accurately 4.74 Gm. of reagent grade potassium aluminum sulfate, $KAl(SO_4)_2 \cdot 12H_2O$, minimum assay 99.5%, dissolve in water, and dilute with water to make 1 liter. Each milliliter of this solution contains 4.74 mg. of alum.

Sodium Citrate, 0.1% Solution.—Dissolve 1 Gm. of reagent grade sodium citrate in about 500 ml. of water, add 8 ml. of concentrated hydrochloric acid, mix thoroughly, and make up to 1 liter with water.

Preparation of Sample

Samples are customarily submitted by the standardization unit. These vary in volume from 25 to 100 ml. Take an aliquot, after shaking the sample to distribute the precipitate as evenly as possible, and transfer to a glass-stoppered flask. Add an equal volume of the 1% acid-citrate solution. Allow the mixture to stand after shaking until it clears up and becomes brilliant. About fifteen minutes is generally required for this. The clear solution is now ready for analysis.

Samples to which citrate solution has already been added are ready to be tested, if they are in complete solution.

Procedure

Transfer 20 ml. of the prepared solution of alum-precipitated toxoid in acid-sodium citrate solution to a 125-ml. Erlenmeyer flask. Add 3 drops of phenolphthalein indicator solution and 20 ml. of 30% potassium sodium tartrate solution. Mix gently and neutralize with saturated barium hydroxide solution to a definite pink end point. Add 20 ml. of 30% potassium fluoride solution and mix. Allow the test solution to stand for five minutes. Titrate with the standardized 0.03 *N* hydrochloric acid until the pink coloration is discharged and remains discharged for thirty seconds.

To calculate the mg. of alum per ml., multiply the milliliters of 0.03 *N* hydrochloric acid used by 4.74 or the equivalent factor and divide by the volume of toxoid taken for the analysis (in this instance 10 ml. of toxoid in 20 ml. of the prepared test solution).

RESULTS

Sixteen 20-ml. aliquots of the standard alum solution were titrated according to the procedure detailed above. The minimum value for mg. alum per ml. obtained was 4.54, the maximum value was 4.91, and the average value was 4.76. In comparison, seven determinations made by the gravimetric method of Berger (3) had a minimum value of 4.52, a maximum value of 4.84, and an average value of 4.65 mg. alum per ml. The calculated value was, as mentioned, 4.74.

Five batches of alum-precipitated toxoid (4 diphtheria toxoid and 1 tetanus toxoid) were analyzed by both the rapid acidimetric and gravimetric method. In all instances the result for the gravimetric procedure was higher than that of the titrimetric procedure. Since the results on the standard alum solution were so close by both methods it was reasonable to infer that the gravimetric method was in error. Qualitative tests showed that in the gravimetric method iron was co-precipitated with the aluminum, if no attempt was made to remove the iron before precipitation of the aluminum. It was deemed simpler for our purposes to estimate the amount of iron in the aluminum phosphate precipitate by the ferric thiocyanate method and then correct for the amount of iron phosphate in the ash of the gravimetric method. These results are shown in Table I. A more extensive series of tests on another toxoid are shown in Table II.

The ash of the gravimetric determinations was dissolved in hydrochloric acid diluted to 50 ml. and iron was estimated as mentioned by the thiocyanate method.

TABLE I.—COMPARISON OF GRAVIMETRIC AND ACIDIMETRIC METHODS FOR ALUM IN DIPHTHERIA TOXOID

Sample No.		Acidimetric, Alum/Ml.	Gravimetric, Direct, Mg. Alum/Ml. (Apparent)	Iron Correction, Mg.	Gravimetric, Mg. Alum/Ml. (Corrected)	Difference between Corrected Gravimetric and Acidimetric Values, Mg.
H1	1	7.38	8.73	1.05	7.68	+0.30
	2	7.28	8.30	0.87	7.43	+0.15
	Av.	7.33	8.51	0.96	7.55	+0.22
H2	1	7.09	7.93	0.95	6.98	−0.11
	2	7.38	7.93	0.95	6.98	−0.42
	Av.	7.23	7.93	0.95	6.98	−0.25
H3	1	7.14	8.12	0.96	7.16	+0.02
	2	7.21	8.35	0.96	7.39	+0.18
	Av.	7.17	8.24	0.96	7.28	+0.11
H4	1	7.18	7.91	0.94	6.97	−0.21
	2	7.14	7.89	1.00	6.89	−0.25
	Av.	7.16	7.90	0.97	6.93	−0.23

TABLE II.—COMPARISON OF GRAVIMETRIC AND ACIDIMETRIC METHODS FOR ALUM IN TETANUS TOXOID

Acidimetric,[a] Mg. Alum/Ml.	Gravimetric,[a] Direct, Mg. Alum/Ml. (Apparent)	Iron Correction, Mg.	Gravimetric, Mg. Alum/Ml. (Corrected)	Difference between Corrected Gravimetric and Acidimetric Values, Mg.
13.43	15.16		14.17	
13.62	14.96		13.96	
13.86	15.28		14.26	
13.53	15.55		14.55	
14.10	15.11		14.10	
13.51	15.23		14.24	
14.20	15.20		14.20	
13.69	15.16		14.17	
13.19	15.01		14.01	
13.28	15.04		14.02	
13.55	15.15		14.12	
Av. 13.66	15.17	1.01	14.16	+0.50

[a] Columns 1 and 2 represent individual aliquots of the original sample diluted at different times, therefore the difference between the corrected gravimetric and acidimetric values can only be obtained on the average.

Calculation of Correction

To calculate the correction the following steps were performed. The amount of iron in 1 ml. of the dissolved ash was estimated. This value was multiplied by 50 to give the total amount of iron in the ash. This must be evaluated in terms of ferric or ferrous phosphate. Since ferrous phosphate is more insoluble, this was chosen although there is actually little difference in the correction whichever iron phosphate is assumed to be present. Hence

$$\text{mg. Fe} : 55.85 :: X : 189.96$$

X is thus the mg. of ferrous phosphate. Subtract this from the total weight of the ash, that is the apparent aluminum phosphate. Multiply the latter by 3.89 and divide by the sample taken for analysis to give the mg. alum per ml. of toxoid.

DISCUSSION

The theoretical aspects of the titrimetric method for the estimation of aluminum by the methods of Viebock and Brecher and of Snyder have been discussed by these authors and need no repetition here. Of greater moment is the fact that the unmodified method of Berger gives erroneous results that are obviated by use of the acidimetric method. The alizarin red method also gives erroneous results, as indicated by Underhill and Peterman (11) for they eliminated the iron interference by a prior extraction of ferric thiocyanate with ether.

Care must be taken to wait for five minutes after the addition of the potassium fluoride solution and before the titration, otherwise difficulty is encountered in obtaining the correct end point. There is some liberation of alkali for a time after the addition of the fluoride solution since the reaction is not complete instantaneously.

If the length of time required for the preparation of sample is not counted, since samples have to be prepared for all methods, the titrimetric is the most rapid method for only about ten minutes is required for a determination. The acidimetric method lends itself to routine analysis. It is, as has been clearly shown, less subject to error than the gravimetric method if correction of the iron error is not made. The precision is adequate for the purpose at hand.

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Microtoxicology. VIII. Optical Crystallographic Properties and Microchemical Reactions of Several Long-Acting Antihistaminic Drugs*

By THOMAS J. HALEY† and GEORGE L. KEENAN‡,§

Microchemical tests have been applied to twelve new antihistamine drugs, and their optical crystallographic properties have been determined. The results of these studies are reported. The conclusions reached are that while microchemical reactions are useful as confirmatory tests, optical crystallographic data offer the best means for rapid identification.

CHEMICAL, pharmacological and clinical research groups are making a concerted effort to develop antihistaminic drugs having a longer duration of action than those currently available (14). This has resulted in the synthesis of Thenfanil, Win-2842 [N′-(2-pyridyl)-N′-(3-thenyl)-N,N-dimethyl ethylenediamine HCl] (1, 2); Chloro-Trimeton Maleate [1-(4-chlorophenyl)-1-(2-pyridyl)-3-,N,N-dimethylpropylamine maleate] (3); *p*-Fluorobenzyl D.P.E. [N′-(4-fluorobenzyl)-N′-(2-pyridyl)-N,N-dimethylethylenediamine HCl] (4); Perazil, Chlorocyclizine [N-methyl-N′-(4-chlorobenzhydryl)-piperazine di HCl] (5–7); AH-289 [Di Paralene, Perazil mono-HCl] (8); AH-853 [*β*-(4-methylbenzhydryl)-N,N-dimethylethanolamine HCl] (9); Anthallan HCl [3-di-(*n*-butyl) aminomethyl-4,5,6-trihydroxy-phenylphthalide HCl] (10); Phenergan HBr, Lilly-01516 [10-*β*-dimethylaminopropylphenothiazine HBr]; Lilly-01798 [1-(10-acridyl)-2-dimethylaminoethane HCl] (11, 12); Lilly-01003 [1-piperidino-3,4-diphenylbutene-3 HCl]; and Bristol C-5581-H [2-benzylphenoxy-N,N-dimethylethanolamine dihydrogen citrate] (13).

In keeping with our earlier work (14–18) we have compared the reactions of these newer drugs with the methods and reagents used previously. Furthermore, we have investigated the reactions of Antergan HCl [N′-(phenyl)-N′-(benzyl)-N,N-dimethylethylenediamine HCl] because this drug is widely used in Europe and little has been published concerning its identification (19).

EXPERIMENTAL

The melting points of the drugs given in Table I were determined with a Melt-Pointer electrically heated melting point apparatus using Anschutz thermometers and capillary tubes. All melting points are uncorrected.

TABLE I.—MELTING POINTS OF THE LONGER-ACTING ANTIHISTAMINICS

Drug	Derivative	Melting Point, °C.
Thenfanil	Hydrochloride	150–151.5
Chloro-Trimeton	Maleate	128–130
p-Fluorobenzyl D. P. E.	Hydrochloride	161–162
Perazil	Dihydrochloride	208–214 dec.
AH-289 Abbott	Hydrochloride	214.5–217 dec.
AH-853 P. D. Co.	Hydrochloride	146–147
Anthallan	Base	52.5 (Ref. 10)
	Hydrochloride	79–80
Phenergan	Hydrobromide	210–211
Lilly-01798	Hydrochloride	206–207
Lilly-01003	Hydrochloride	240–244
C-5581-H Bristol	Dihydrogencitrate	130–130.5
Antergan	Hydrochloride	199–200

The reagents were prepared as directed by Thienes and Haley (20). The compounds were tested by placing one drop of reagent on a microscope slide or in the well of a spot-plate and adding about 0.1 mg. of the drug to it. The results of this addition were observed for approximately thirty minutes and the changes taking place recorded. In the case of the acidic colorimetric reagents, the end point was the formation of a stable color or a charring of the drug, but only the colors appearing prior to this were used for identification purposes. In the case of the precipitation reagents, those which formed definite crystalline precipitates were set aside until dry, and, where possible, the crystals were examined for their optical crystallographic properties by the immersion method. Table II gives the results of the colorimetric and precipitation tests.

Inasmuch as the colorimetric and precipitation tests were helpful but not conclusive, the optical crystallographic properties of the pure compounds were studied by the immersion method. These optical crystallographic properties are given in Table III. Furthermore, photomicrographs of the

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TABLE II.—COLORIMETRIC AND PRECIPITATION REACTIONS OF THE LONGER-ACTING ANTIHISTAMINICS

Reagent	AH-289 HCl Perazil Di HCl	Chloro-Trimeton Maleate	p-Fluorobenzyl D. P. E. HCl	Thenfanil HCl	AH-853 HCl	Anthalan HCl	Antergan HCl	Phenergan HBr	Lilly 01798 HCl	Lilly 01003 HCl	Bristol C-5581-H Dihydrogen Citrate
Concd. H_2SO_4	Canary-yellow	Colorless	Effervescence Cloudy solution	Brilliant carmine	Yellow with orange-red oily globules	Pale brown	Effervescence Colorless	Fuschia	Greenish yellow	Brown	Faint pink
Concd. HNO_3	Greenish yellow	Colorless	Colorless	Reddish orange	Cloudy solution	Orange	Pink with purple center	Bloody red rapidly fading	Greenish yellow	Colorless	Purple
Mandelin's	Reddish orange	No change	Orange with greenish spots	Orange-brown to deep brown	Orange with orange-red oily globules	Gray to faint lavender	Orange	Magenta to purple with blue center	Red	Brown	Emerald-green to brown to orange
Marquis'	Canary-yellow	No change	Pale pink	Magenta	Orange with red globules to brown	Pale brown	No change	Carmine	Yellow to greenish yellow	Brown	Carmine
Buckingham's	Canary-yellow	Colorless	Effervescence Cloudy solution	Brilliant carmine to magenta	Orange with oily red globules	Deep blue	Effervescence Colorless	Magenta changing to brown	Green	Dark blue	Dirty green, faint pink, finally orange
Fröhde's	Canary-yellow	Colorless	Effervescence Cloudy solution	Brilliant carmine	Greenish yellow	Gray, pink outline with greenish center	Reddish orange	Mangeta	Yellow to greenish yellow	Canary-yellow	Faint pink
Chloroauric acid	Amorphous precipitate	Bundles of needles in burr-like agglomerates	Amorphous precipitate	Amorphous precipitate	Thin, narrow rods, blunt ends	Amorphous precipitate	Red amorphous precipitate	Black amorphous precipitate	Green color, fine needles, then larger rods	Amorphous precipitate	Amorphous precipitate
Chloroplatinic acid	Amorphous precipitate	Amorphous precipitate	Bundles of fine needles in burr-like agglomerates	Black amorphous precipitate	Amorphous precipitate	Amorphous precipitate	Irregular flat plates	Green color then brown amorphous precipitate	Fading red color, amorphous precipitate	Amorphous precipitate	Amorphous precipitate
Satd. soln. of picric acid	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate

TABLE III.—OPTICAL CRYSTALLOGRAPHIC PROPERTIES OF ANTIHISTAMINICS

Substance	Habit	Extinction	Elongation	2E	Optic Sign	Refractive Indices α	β	γ
Perazil Di HCl	Variable	Parallel	...	Small	−	1.610	1.660	1.665
Di-Paralene HCl	Plates	−	...	76° ± 5°	+	1.590 ±0.002	1.610 ±0.002	1.665 ±0.005
Chloro-Trimeton Maleate	Prisms	−	...	−	...	1.533 ±0.002	1.668 ±0.002	<1.734 ±0.002
p-Fluorobenzyl HCl	Rods, plates	Parallel	−	Large	+	1.585 ±0.002	1.600 ±0.002	1.668 ±0.002
Thenfanil HCl	Plates, prisms	−	...	Large	...	1.590 ±0.002	[a]Indt. ...	1.680 ±0.002
AH-853 HCl	Plates	Inclined	±	Small	−	1.562 ±0.002	1.606 ±0.002	1.610 ±0.002
Anthallan HCl	Rods	Parallel	−	−	...	1.505 ±0.002	1.585 ±0.002	1.617 ±0.002
Antergan HCl	Prisms	−	...	−	+	1.587 ±0.002	1.635 ±0.002	1.734 ±0.005
Phenergan HBr	Prisms	−	...	Small	+	1.667 ±0.002	1.675 ±0.002	>1.733 ±0.002
Lilly-01798 HCl	Plates	Parallel	−	−	...	1.617 ±0.002	Indt. ...	>1.733 ±0.002
Lilly-01003 HCl	Plates	−	...	−	+	1.620 ±0.002	1.636 ±0.002	1.667 ±0.002
C-5581-H Dihydrogen Citrate	Rods	Inclined	...	−	−	1.556 ±0.002	1.585 ±0.002	1.610 ±0.002

[a] Indt. = indeterminant

pure compounds were taken with an E. and G. Visicam photographic attachment on a B. and L. monocular microscope using the 16-mm. objective and the 10× eyepiece. Figures 1–12 show typical crystals of the pure compounds and may be used as a further means of identification.

Perazil Dihydrochloride (Fig. 1) is unique in that the habit of the crystals is so variable, consisting of short rods, irregular fragments, and elongated six-sided plates. The α refractive index is shown crosswise on the rods and irregular fragments, while the γ value is shown lengthwise on both. The γ value is the most frequently found index.

Abbott AH-289, Di Paralene (Fig. 2) is composed of two types of crystals—thin, plate-like and thicker, irregular fragments. The former are the most significant. With parallel polarized light the thin fragments show low order white colors, while the thicker ones exhibit brilliant polarization colors.

Chloro-Trimeton Maleate (Fig. 3) consists of irregular fragments and elongated box-like prisms closely resembling forms characteristic of the orthorhombic system. The lack of interference figures on the substance precludes definite orientation in respect to the refractive indices. Therefore, the

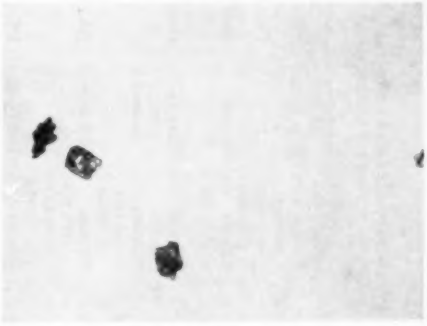

Fig. 2.—Abbott AH-289, Di Paralene.

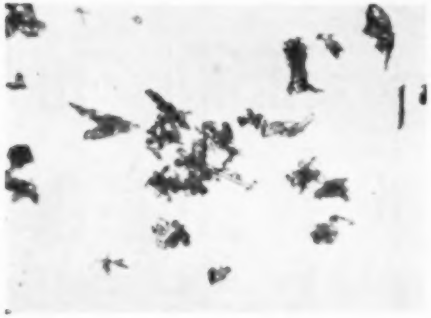

Fig. 1.—Perazil Dihydrochloride.

Fig. 3.—Chloro-Trimeton Maleate.

minimum refractive index shown on fragments with the double refraction is considered as *alpha*. *Beta* could not be definitely identified, but an intermediate value, 1.668, occurs frequently. The values for α and β (see Table III) are readily found and are significant.

p-Fluorobenzyl D. P. E. Hydrochloride (Fig. 4) consists of rods, square plates, irregular fragments, and some rods with obtuse terminations. The substance is apparently orthorhombic. The α refractive index is shown lengthwise on the rods while both the β and γ values are shown crosswise on the rods. These indices are readily found.

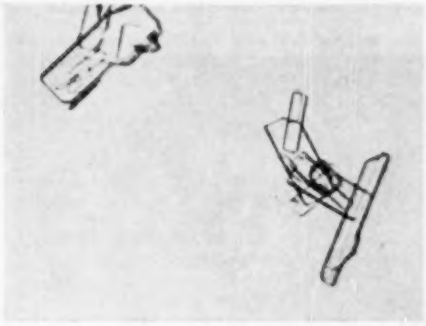

Fig. 4.—*p*-Fluorobenzyl D. P. E. Hydrochloride.

Thenfanil Hydrochloride (Fig. 5) consists of square plates, small stubby prisms, and irregular fragments. Interference colors are brilliant under parallel polarized light, but many fragments do not extinguish sharply when the stage is revolved. This is characteristic of this compound. In convergent polarized light, partial biaxial interference figures showing the isogyres at the edge of the field are common. The minimum refractive index is considered as α and it occurs infrequently. The maximum value which occurs more frequently is considered as γ. The β value could not be determined due to the absence of a suitable interference figure.

Fig. 5.—Thenfanil Hydrochloride.

Parke-Davis AH-853 Hydrochloride (Fig. 6) consists of rectangular plates, some of which are isodiametric and elongated, while others are irregular fragments. Many of these fragments do not extinguish sharply under parallel polarized light.

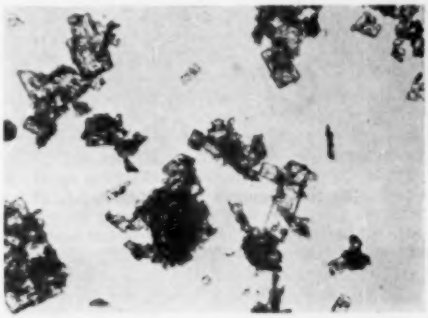

Fig. 6.—Parke-Davis AH-853 Hydrochloride.

Anthallan Hydrochloride (Fig. 7) consists of small rods and irregular fragments. Under parallel polarized light most fragments extinguish sharply, but under convergent polarized light no interference figures were observed.

Fig. 7.—Anthallan Hydrochloride.

Antergan Hydrochloride (Fig. 8) consists of small, short, clear, glassy prisms which upon crushing gives irregular fragments without definite habit. Under parallel polarized light many fragments extinguish sharply and others remain bright when the microscope stage is revolved. Under convergent polarized light, partial biaxial interference figures occur with the optic axis at the extreme edge of the field. The refractive index values α and β are common, while the γ value is uncommon.

Phenergan Hydrobromide (Fig. 9) consists of massive prisms, some short and stubby and others elongated. Under parallel polarized light many fragments extinguish sharply, while others remain bright when the microscope stage is revolved. The

Fig. 8.—Antergan Hydrochloride.

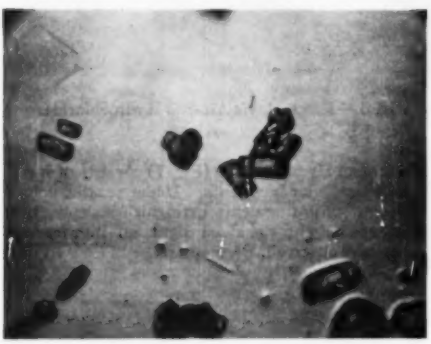

Fig. 9.—Phenergan Hydrobromide.

Fig. 10.—Lilly-01798 Hydrochloride.

refractive index values α and β are especially significant for this compound.

Lilly-01798 Hydrochloride (Fig. 10) consists of brownish, thin plates, some elongated and six-sided in outline, frequently with dagger-shaped acute terminations. Under parallel polarized light the plates extinguish sharply while the elongated forms show parallel extinction and negative elongation. Under convergent polarized light, interference figures are difficult to locate, although the substance is apparently biaxial. Although the β refractive index could not be determined, an intermediate value, 1.636, occurs frequently.

Lilly-01003 Hydrochloride (Fig. 11) consists of thin, rectangular, overlapping plates or scales with a pearly luster. Many plates do not extinguish sharply under parallel polarized light. Under convergent polarized light the optic axis figures are significant. The orientation of the plates is such that the common refractive index values are β and γ. The α value can be obtained by finely powdering the plates.

Bristol C-5581-H Dihydrogen Citrate (Fig. 12) consists of rods and needles which rarely show interference figures under convergent polarized light.

Fig. 11.—Lilly-01003 Hydrochloride.

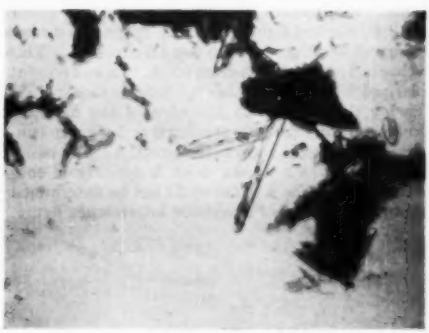

Fig. 12.—Bristol C-5581-H Dihydrogen Citrate.

DISCUSSION

Both AH-289 and Perazil give general color reactions similar to those reported for other antihistaminics (15–18) and alkaloids (21) and their reaction with Mandelin's reagent is the same as that of the thenyl antihistaminics with the exception of Thenfanil. However, all of the thenyl series drugs react differently with the other reagents so that Mandelin's reagent would serve as a confirmatory test for both AH-289 and Perazil.

Chloro-Trimeton is extremely hard to identify microchemically because of its unreactive nature being similar to Trimeton in this respect (16). However, the crystalline precipitate produced with chloroauric acid and the amorphous precipitate produced with picric acid serve to differentiate Chloro-Trimeton from Trimeton because the latter gives a crystalline precipitate with picric and not chloroauric acids (16).

The reaction between Mandelin's reagent and *p*-Fluorobenzyl D. P. E. differs from any previously reported (15–18, 21) and thus would serve to identify this compound. Further confirmatory evidence could be obtained from the fact that a crystalline precipitate is formed with chloroplatinic acid. Only Pyribenzamine (14), Histadyl (16), and Decapryn (16) of all the antihistaminics tested give crystalline precipitates with this reagent and the crystals are sufficiently different so that no trouble in identification would be encountered.

The reactions of Thenfanil differ slightly from those reported for the other thenyl antihistaminics (16) but direct side-by-side comparison would show the differences. Although all of the thenyl compounds give amorphous precipitates with chloroplatinic acid, only Thenfanil gives a black color with this reagent so that the reaction could be used as a final identifying test.

The reactions between AH-853 and concentrated sulfuric acid, Mandelin's, and Buckingham's reagents are identical with those reported for Benadryl, Linadryl, and No. 204 and are probably due to the ether linkage between the benzhydryl and the ethylamine portions of the molecule (16). The reaction between AH-853 and Fröhde's reagent is very similar to that observed with Thephorin, Sc 887, and colchicine (16, 21) but the other tests would serve to differentiate these compounds from one another.

Anthallan's reaction with concentrated sulfuric acid is similar to that given by emetine and narceine (21) but these latter compounds differ in their reactions to the other reagents so no difficulty should be encountered in differentiating between them. Concentrated nitric acid may be useful but Bromothen, Tagathen, codeine, and papaverine (16, 21) also produce colors resembling that given by Anthallan. The reactions between Anthallan and Mandelin's and Fröhde's reagents differ from any previously reported so that these are the most useful reagents for identifying the compound.

Concentrated nitric acid is the only reagent giving a distinctly different color reaction with Antergan, all the other color tests resembling those reported for either antihistaminic compounds (15–18) or alkaloids (21). The precipitation reactions between Antergan and chloroauric and chloroplatinic acids would also aid in identifying this compound.

Phenergan Hydrobromide reacts differently than its hydrochloride salt with all of the reagents so that no difficulty would be encountered in differentiating between the two compounds (18). However, some difficulty might be encountered in differentiating the hydrobromide salt from the other compounds of the thiodiphenylamine series. Mandelin's reagent is the most useful in differentiating Phenergan Hydrobromide from the other thiodiphenylamine antihistaminics.

Although Antistine (16), atropine, brucine, morphine, narcotine, and veratrine (21) give red colors similar to that seen when Lilly-01798 reacts with Mandelin's reagent, the other tests would serve to differentiate between these compounds. Lilly-01798, codeine (21), Thephorin, and Sc 887 (16) give a green color with Buckingham's reagent, but no difficulty should be encountered in differentiating between these compounds. The green color and the crystalline precipitate obtained with chloroauric acid as well as the red color obtained with chloroplatinic acid would serve as a confirmatory test in identifying Lilly-01798.

The colorimetric reactions of Lilly-01003 are similar to those previously reported for many alkaloids (21) and side-by-side comparative tests would be necessary for identifying this antihistaminic.

The reactions of C-5581-H and concentrated sulfuric acid, concentrated nitric acid, Mandelin's, Buckingham's, and Fröhde's reagents are sufficiently different from any previously reported for either the antihistaminics (15–18) or the alkaloids (21) that the tests would aid in identifying C-5581-H. In addition, the reaction between Marquis' reagent and C-5581-H is only duplicated by 929F (16) so that this test could also be used to differentiate C-5581-H from many compounds.

Although the colorimetric and precipitation reactions of this group of antihistaminics are useful as confirmatory tests, their optical crystallographic properties offer the best evidence in their identification.

SUMMARY

Twelve new antihistaminic drugs were investigated for their reactions to microchemical tests and for their optical crystallographic properties. The compounds are most readily identified and differentiated by use of their optical crystallographic properties. These properties should prove quite useful to chemists as specific tests for identifying these new antihistaminics.

p-Fluorobenzyl D. P. E. Hydrochloride may be identified by its reaction with Mandelin's reagent. Antergan Hydrochloride may be differentiated from both the other antihistaminics and various alkaloids by its reaction with concentrated nitric acid. Phenergan Hydrobromide reacts with most of the reagents differently from the other thiodiphenylamine antihistaminics so that it may be differentiated from the other drugs of that series. Of all the compounds investigated, C-5581-H dihydrogen citrate is most readily identified with the colorimetric reagents used in microtoxicological investigations.

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A Colorimetric Method for the Estimation of Colchicine in Pharmaceutical Preparations*

By HARRY MACK and EDWARD J. FINN

A former qualitative test for colchicine has been made the subject of a quantitative study in this report. Although the test is not specific for the drug in the presence of aldehydes and ketones, it is, however, serviceable in mixtures with inorganic salts and other organic substances from which colchicine can be quantitatively separated.

DESPITE the fact that the determination of colchicine in pharmaceutical preparations has been made the subject of numerous investigations, an extensive literature survey reveals that a desirable colorimetric procedure for the estimation of trace quantities is still lacking. Since the average dose of colchicine varies between 0.5 to 1.2 mg. ($^1/_{120}$ to $^1/_{50}$ gr.) and since many marketed preparations contain even less because of the desire of the manufacturer to stay well within the margin of safety, a rapid, satisfactory method for routine control without the use of cumbersome equipment, would be welcomed. In an effort to resolve some of the difficulties we have experienced, the present series of investigations was undertaken.

The U. S. P. XIII (1) currently outlines a gravimetric method for colchicine tablets wherein a number of tablets, equivalent to 50 mg. of colchicine, are extracted and weighed. A modification of the U. S. P. procedure is that of Webber (2) who applied the use of lead subacetate to the concentrated alcoholic tincture to precipitate resin acids and then proceeded according to the method outlined in the U. S. P. XII for colchicum seed. The British Pharmacopœia (3) under colchicum seed also indicates an extraction procedure followed by weighing the residue. Self and Corfield (4) describe a similar method of determination based upon the "salting-out" effect of sodium sulfate in solutions of colchicine and using ethyl ether to extract the drug. These methods are poorly suited from a manipulative standpoint, however, to determine quantities of colchicine in the concentration of a milligram or less per unit of weight or volume. It is, perhaps, because of these shortcomings that a few investigators have turned toward some recent applications of absorption chromatography for solution of some of the experimental difficulties encountered in the gravimetric analysis. Muhleman and Tobler (5) applied a chromatographic-titrimetric procedure to the alkaloids in the tinctures of colchicum corm and seed of the Swiss Pharmacopœia. The method is based upon the adsorption of the colchicine on aluminum trioxide; elution with 50–70 per cent alcohol and the subsequent reaction with iodine to form the alkaloidal hydriodide salt. Another method that has been suggested by Goursat (6) depends upon the dissociation of the salts of colchicine. A photometric method has been applied by Seifert (7) to colchicine involving the production of a green color in the Zeisel determination. The procedure requires only a small sample.

The reaction which we have developed is an outgrowth of a qualitative test first noted by Kippenberger (8) in 1897. When hydroxylamine hydrochloride and sodium hydroxide in solution are added in slight excess to an aqueous solution of colchicine, an orange color develops on standing and especially upon warming the solution. After standardizing the reagent solutions and the reaction time, what first appeared to be a quali-

* Received October 18, 1949, from the Analytical Laboratories of The National Drug Co., Philadelphia.

tative test later developed into a satisfactory rapid quantitative reaction resulting in a stable color which could be read in any photometer at a wave length of 500 mμ. The substances most likely to interfere with the test are aldehydes and ketones and in their presence an extraction method must be chosen to avoid their inclusion in the reaction solution.

In the early stages of the investigation the preliminary work was done with the Klett-Summerson Photoelectric Colorimeter. Filter No. 50 was selected as most satisfactory. By plotting the extinction values against concentration, a linear relationship was found to exist. However, when the absorption spectra of a 3-mg. solution of colchicine were measured with the Beckman Spectrophotometer from a wave length of 320 to 620 mμ, maximum extinction was discovered at 340 mμ with only a 0.6 per cent transmission. It became apparent that it would be prohibitive to make accurate readings at this wave length. From an analytical point of view some wave length had to be found which showed agreement with Beer's law within the working concentrations and corresponded to densities between 0.022 and 1.0. After some experimentation 500 mμ was found to satisfy the experimental conditions.

EXPERIMENTAL

Preparation of the Standard Graph.—For stability reasons it was found more advantageous to use pure colchicine salicylate for the reference standard rather than the alkaloid and to conduct all the experimental investigations in subdued light. The colchicine salicylate assayed 74.13% colchicine by the gravimetric method using 120 mg. of the material for extraction.

Of this pure salt, 40.5 mg. was accurately weighed, dissolved in 75 ml. of warm water in a 100-ml. volumetric flask and after cooling to 25° the volume of solution was adjusted to the mark. Quantities of this dilution were accurately measured to yield 1, 2, 3 and 4 mg. of colchicine and diluted with sufficient water to give exactly 15 ml. of solution contained in 25-ml. test tubes. A tube containing 15 ml. of distilled water served as a blank and was subjected to the same treatment as the standard colchicine tubes.

To each test tube was added 2 ml. of a 2% aqueous solution of hydroxylamine hydrochloride and 1.5 ml. of half-normal sodium hydroxide. Each tube was mixed thoroughly and all tubes were simultaneously heated for exactly five minutes in a water bath maintained at 75°. The tubes were removed and chilled in an ice bath to about room temperature and read in 1-cm. cells in the Beckman Spectrophotometer at 500 mμ with a 0.2-mm. slit opening. The values that were obtained are shown in Table I and plotted in Fig. 1. Adherence to Beer's law is indicated.

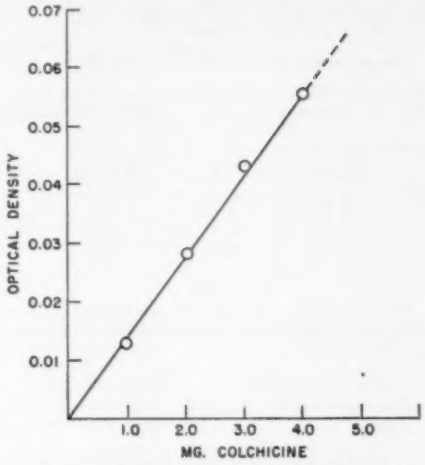

Fig. 1.—Standard graph obtained by plotting the average values shown in Table I.

TABLE I.—RELATIONSHIP BETWEEN SPECTROPHOTOMETRIC READINGS AND CONCENTRATION

Concentration Mg. Colchicine	Per Cent Transmission	Optical Density
Blank	(*a*) 100.0	0.00
	(*b*) 100.0	0.00
1 Mg.	(*a*) 97.0	0.013
	(*b*) 97.0	0.013
2 Mg.	(*a*) 94.0	0.027
	(*b*) 93.5	0.030
3 Mg.	(*a*) 90.0	0.046
	(*b*) 91.2	0.040
4 Mg.	(*a*) 88.0	0.056
	(*b*) 88.2	0.055

In order to determine how well the procedure operates for small amounts of colchicine in aqueous preparations, a solution was prepared containing:

Colchicine (as Salicylate)....0.5 gr. (32.4 mg.)
Sodium Iodide.....775.0 gr. (50.2 Gm.)
Sodium Salicylate.....775.0 gr. (50.2 Gm.)
Distilled water, a sufficient quantity, to make.....1000.0 ml.

A 100-ml. portion of the solution was accurately measured into a 250-ml. Squibb type separatory funnel, made just alkaline to litmus with about two drops of half-normal sodium hydroxide and extracted with three successive 25-ml. quantities of chloroform. The combined solvent was evaporated to dryness on the steam bath with the aid of air. The residue was taken up with 10 ml. of warm water, transferred to a 25-ml. test tube graduated to 15, ml., 1.0 mg. of sodium salicylate in solution added, and the mixture adjusted to the mark with water. The reaction was run in the manner previously indicated concurrently with a blank containing 3 mg. colchicine as the salicylate and the milligram concentration of colchicine in the aliquot was calculated from the ratio of the optical densities (p. 534):

$$\frac{E_{500}\text{ sample}}{E_{500}\text{ standard}} \times 3$$

The recoveries are presented in Table II.

TABLE II.—RECOVERY OF COLCHICINE FROM SOLUTION IN COMBINATION WITH SODIUM SALICYLATE AND SODIUM IODIDE

Mg. Added (100 Ml.)	Mg. Recovered	Per Cent Recovered
3.24	3.56	110
3.24	3.56	110
3.24	3.56	110
3.24	3.56	110

We attempted to apply the procedure to tablet and powder mixtures of which the following formula may be typical:

Colchicine (as Salicylate).... 1.39 gr. (90 mg.)
Potassium Iodide.....231.50 gr. (15.0 Gm.)
Talc.....462.90 gr. (30.0 Gm.)

A suitable quantity, which in this case was 7.5 Gm. of powder, was extracted directly with 20 ml. chloroform, allowed to settle, and the solvent decanted into a filter. The extraction was repeated four successive times and the filter washed well with chloroform. The combined solvent was evaporated to dryness and the residue dissolved in 15 ml. of boiling water and transferred to a 50-ml. volumetric flask. This operation was repeated twice more and after cooling the combined aqueous extracts to room temperature, the volume was adjusted to the mark with distilled water. To a 10-ml. aliquot, accurately measured, and contained in a test tube graduated at 15 ml., 1.0 mg. of sodium salicylate was added in solution, the mixture diluted to volume, the color developed, and the results calculated by the method previously explained. The results are given in Table III.

TABLE III.—RECOVERY OF COLCHICINE FROM A MIXTURE IN COMBINATION WITH SODIUM IODIDE AND TALC

Mg. Added (in 1.5 Gm.)	Mg. Recovered	Per Cent Recovered
3.0	2.9	96.6
3.0	2.9	96.6
3.0	2.9	96.6
3.0	2.9	96.6

DISCUSSION

Mention was made before of the unaccountable difficulty that was experienced in the recovery of colchicine from solutions in the absence of salicylates. The results were variable and constantly higher than we found by using colchicine salicylate. However, in each case, by the addition of sodium salicylate it was possible to achieve consistent results. Consequently it is suggested that when colchicine alkaloid is assayed by this technique, it is advisable to conduct the determination in the presence of salicylates before developing the color. Table IV

TABLE IV.—RECOVERY OF COLCHICINE BY COLOR DEVELOPMENT IN THE PRESENCE OF SODIUM SALICYLATE

Mg. Colchicine Alkaloid Added	Mg. Sodium Salicylate	Mg. Colchicine Recovered
1.0	0.3	1.05
2.0	0.6	2.20
3.0	(*a*) 1.0	2.90
	(*b*) 1.0	2.90
	(*c*) 1.0	2.90
4.0	1.2	4.00

illustrates the recoveries of colchicine to which sodium salicylate has been added prior to color development. Note that, within experimental error, the results agree well with the theory and are exceedingly reproducible.

SUMMARY

1. A simple, rapid, colorimetric determination of small amounts of colchicine in solution and powder formulations is presented.

2. The results are reproducible and the recoveries, within experimental error, are good.

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Biological Safety Tests for the Quality Control of Parenteral Pharmaceuticals*

By NATHAN RAKIETEN†

Biological safety tests are proposed for parenteral water-soluble drugs and for drugs in oil suspensions. The test dosages are based on the intravenous or intramuscular administration of the drugs in doses which approach or surpass symptomatic doses in the species of animal used. The tests thus aid in detecting serious departure from proper compounding procedures and are suggested as routine control measures in the manufacture of the enumerated drugs.

BIOLOGICAL safety tests for parenteral pharmaceuticals are important safeguards in their standardization and quality control. Such tests provide biological evidence for the presence of the active ingredients and a rough index of their concentration. While such tests are standard practice for antibiotics, they have only recently been suggested by Brewer (1) for drugs other than those few for which biological standardizations are described in the U. S. P. The tests described in this paper were devised in 1947, and their presentation at this time following Brewer's publication (1) provides additional information concerning their application and scope.

The tests described herein were set up empirically to suit best the needs of our laboratories. They are based on the intravenous or intramuscular administration of the drugs in doses which approach or surpass symptomatic doses in the species of animals used. In this study albino mice (R. A. P. strain) and albino rats (Sherman strain) were used exclusively. Dosages for mice such as those used in this study, provide high factors of safety and insure that their gross effects will be evident, i.e., excitement, depression, or anesthesia with the barbiturates, hyperactivity and convulsions with caffeine and sodium benzoate, and hyperactivity, incoordination, and convulsions with procaine hydrochloride. However, some drugs (ascorbic acid) even in exceedingly high dosages (400 mg./Kg.) fail to produce any symptoms. In such cases this test provides only negative results but does indicate the absence of any extraneous toxic material. In the rat safety test, dosages below the symptomatic doses appear adequate to give sufficient factors of safety.

* Received April 10, 1950, from Bristol Laboratories, Inc., Syracuse, N. Y.
† The author wishes to thank R. H. Noel, Director of Control, Bristol Laboratories, Inc., for his generous suggestions and advice during the study and for his aid in the preparation of the manuscrupt, and to thank Miss Margaret A. Szaraz and Mrs. Agnes A. Bunnell for their technical assistance.

METHODS

An approximate lethal dose of each of the drugs, with the exception of ascorbic acid and the oil suspensions of progesterone, estrogenic substance, and bismuth subsalicylate, was determined. This value was not necessarily the M. L. D. nor was it a statistically determined lethal dose. It was obtained by giving multiple dilutions of the formulations in several animals until a lethal dose was approximated. Following this the formulations were diluted until a dose was obtained which would produce symptoms, i.e., shock, convulsions, hyperactivity, incoordination, depression, etc., but not death (see Tables I and II). All animals were observed for at least forty-eight hours after the drugs were given intravenously and for seven to twenty-one days when the drugs were given intramuscularly. All doses were given in volumes of 0.5 cc. or less.

Following the orientation experiments described above, the following test methods were arrived at for purposes of routine control:

Mouse Safety Test for Water-Soluble Drugs (2)

Test Animals.—Five healthy male mice, whose mean weight may vary between 18–25 Gm., but whose individual weights do not vary more than ±1 Gm. from the mean, are used for each test. The animals are housed in groups of five in raised-bottomed, open-mesh cages and are fed a diet of Purina Rabbit Chow Checkers (supplemented by vitamins B and C) and water *ad libitum*.

Testing Procedure.—The formulations are made up to the required concentrations with either sterile water or physiological saline depending upon the original solvent used for the drug and they are administered intravenously. The injection volumes are kept at or below 0.5 cc. and the animals are observed for forty-eight hours after injection. The drug under test is considered satisfactory if none of the animals die within forty-eight hours. If one death occurs a repeat test is done on five additional mice. If no deaths occur in the repeat test the formulation is considered satisfactory; if one or more deaths occur in the repeat test the formulation is considered unsatisfactory. Supportive evidence for this latter conclusion should be obtained by comparing the LD_{50} of the unknown with that of a standard of known quality.

Rat Safety Tests for Formulations Suspended in Oil

Test Animals.—Healthy young adult rats, male or female, weighing at least 250 Gm. are used for the test.

TABLE I.—MOUSE SAFETY TESTS FOR AQUEOUS PARENTERAL PHARMACEUTICALS

Drug Studied	Concn. Mg./Cc. or U.[a]/Cc.	Approximate Lethal Dose I. V., Mg./Kg. or U./Kg.	Symptomatic Dose I. V., Mg./Kg. or U./Kg.	Test Dose for the Drug Preparation I. V., Mg./Kg. or U./Kg
Caffeine and Sodium Benzoate	250	176	58	125
Posterior Pituitary	5	175	25	50
Epinephrine HCl	1	0.40	0.23	0.20
Sodium Iodide and Sodium Salicylate with Colchicine	50	...	..	300
	100	400	350	300
Sodium Phenobarbital	26–150	175	125	125
Procaine HCl	10 and 20	30	27	25
Aminophylline	24 and 240	150	150	100
Ascorbic Acid	20 and 200	...	..	400
Sodium Ascorbate	50 and 200	1374	930	800
Thiamine HCl	50 and 100	60–82	50–60	50
Vitamin B Complex (Parenteral) in terms of Thiamine HCl	10	27.3	18.4	15
Liver Injection, U. S. P.	10 and 15	100	30	30
Liver Injection, Crude, U. S. P.	2	6	5	4
Liver Injection, Crude, U. S. P.	1	5.4	4	4

[a] U. = Units.

TABLE II.—RAT SAFETY TESTS FOR PARENTERALS SUSPENDED IN OIL

Drug Studied	Concn., Mg./Cc.	Symptomatic Dose[a] I. M., Mg./100 Gm.	Test Dose of Formulation I. M., Mg./100 Gm.
Bismuth Subsalicylate in Oil	130	12.0	5.2
Estrogenic Substance in Oil	1	0.4	0.2[b]
	2	..	0.4
Progesterone in Oil	2	..	0.8[b]
	5	..	1.0
	10	5.0	5.0
	25	..	5.0

[a] Symptoms noted were loss of weight and local reactions at the site of injection.
[b] Dose limited by volume of 0.5 cc.

Testing Procedure.—The preparation, in volumes up to 0.5 cc., is administered intramuscularly into the upper leg muscle mass of a single rat using aseptic technique. A negative control is observed concurrently. The animals are weighed immediately preceding the test, the day following the administration of the drug, and twice during the following six days. When the animals are weighed, they are observed for toxic symptoms and for signs of reactions at the site of the injection. If the animal remains in good condition it may be used for another test after a one- to two-week period. The animals are fed Gaines Krunchon (supplemented by vitamins B and C) and water *ad libitum* and are housed in raised-bottomed, open-mesh cages. The preparation is considered satisfactory if it does not cause death, excessive loss of weight (5%), or undue local

TABLE III.—COMPARISON OF AVERAGE HUMAN DOSES TO DOSES USED IN SAFETY TEST WITH APPROXIMATE FACTORS OF SAFETY

Drug Studied	Average Human Dose	Test Dose I. V.	Approximate Factor of Safety
Caffeine and Sodium Benzoate	8.3 mg./Kg.	125 mg./Kg.	15
Posterior Pituitary	0.83 mg./Kg.	50 mg./Kg.	60
Epinephrine HCl	16.7 μg./Kg.	200 μg./Kg.	12
Sodium Iodide and Sodium Salicylate with Colchicine	16.7 mg./Kg.	300 mg./Kg.	9 and 18
Phenobarbital Sodium Solution and Crystals	5 mg./Kg.	125 mg./Kg.	25
Procaine HCl Solution and Crystals	0.3 mg./Kg.	25 mg./Kg.	75
Aminophylline	6 mg./Kg.	100 mg./Kg.	16
Ascorbic Acid	3.3 mg./Kg.	400 mg./Kg.	100
Sodium Ascorbate	3.3 mg./Kg.	800 mg./Kg.	200+
Thiamine HCl	Usually 1 mg./Kg.	50 mg./Kg.	50
Vitamin B Complex (Parenteral) in terms of Thiamine HCl	1.4 mg./Kg.	15 mg./Kg.	10
Liver Injection, U. S. P.	0.3 u./Kg.	30 u./Kg.	100
Liver Injection, Crude, 2 U. S. P. Units	0.3 u./Kg. daily	4 u./Kg.	13
Liver Injection, Crude, 1 U. S. P. Unit	0.3 u./Kg.	4 u./Kg.	13
Bismuth Subsalicylate in Oil	1 cc./60 Kg.	0.1 cc.[a]	240
Estrogenic Substance in Oil	1 cc./60 Kg.	0.5 or 0.25cc.[a]	120
Progesterone in Oil	1 cc./60 Kg.	0.5 or 0.25[a]	120

[a] These dosages are for a 250-Gm. rat and are given I. M.

reaction at the site of injection (edema, ulceration, necrosis, or impairment of the use of the limb).

The essential data for the derivation of the test dosages are presented in Tables I and II.

An attempt to delineate human factors of safety from these data is presented in Table III. These are at best rough approximations and are based on average human dosages. The dangers inherent in comparing human dosages with animal dosages on a weight-to-weight basis are fully recognized, and undue emphasis on the human factors of safety are unwarranted. They do, however, indicate that massive dosages can be given safely to animals when the preparations are properly compounded and serve as a tool to detect serious departure from proper compounding procedures. This, of course, is essentially the sole purpose of the tests.

SUMMARY

1. Biological safety tests for some parenteral water-soluble drugs and some drugs in oil suspensions are described and suggested as routine control measures in their manufacture.

2. Test dosages of various drugs to be tested are tabulated.

3. Human safety factors have been approximated for several of the common parenteral drugs.

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Note

A Note on a Rapid Method for Titrating Microbiological Assay Culture Tubes*

By M. J. HARDWICK and F. S. TAFT

WHEN a multiple series of microbiological assays of nicotinic acid or riboflavin is to be determined, a large number of test tubes are involved requiring a considerable amount of time consumed rinsing test tubes into the flask used for titration. The transfer of the test tube's contents also adds a theoretical source of error. When conducting the test on several samples, the number of test tubes involved becomes numerous and the final titration becomes long and tedious, making it almost imperative that two technicians work together. This new method makes it possible for one technician to determine a great number of titrations in one-half the time required by two persons and eliminating one theoretical source of error, since there is no loss of time and contents required by transferring and rinsing.

For the titrations a small motor with direct pump which will supply a continuous flow of air is used. The air is delivered into the culture tube by means of a hypodermic needle of sufficient length to reach near the bottom of the tube. This hypodermic needle is fastened to the side of the tip of the automatic burette which delivers the 0.1 *N* sodium hydroxide into the tube. Thus it is possible to titrate the microbiologicals directly in the culture tube.

The air is first pumped through a basic, an acidic, and a water wash. Then it is passed through suitable agents to dry it, and then through the pump and needle into the culture tube. There should be a bleeder valve in the air line, so that it is possible to regulate the flow of air. This constant bubbling of air will provide sufficient turbulence to mix the contents of the culture tube, the alkali and bromothymol blue test solution, the indicator called for in the U. S. P. XIII or N. F. IX.

It has been found convenient to use an 18 x 150-mm. test tube for these culture tubes. First, the different amounts of the test solution of the material to be assayed are added, plus 5 cc. of basal medium stock solution, and sufficient water to make 10 cc. To this 10 cc., we have found that titrations of more than 5 or 6 cc. of 0.1 *N* NaOH cannot be relied on, giving us a total of 15 to 16 cc. of solution which can be mixed very well by controlling the amount of air let in. We find this size tube gives us sufficient room to allow a good mixing and to show us a sharp, reproducible end point.

This apparatus (see p. 538, Fig. 1) can be constructed from ordinary laboratory equipment at relatively small expense, and its use makes it possible for one technician to titrate a greater number of microbiological samples in a much shorter period of time.

* Received March 31, 1950, from the Control Laboratory, S. E. Massengill Co., Bristol, Tenn.-Va.

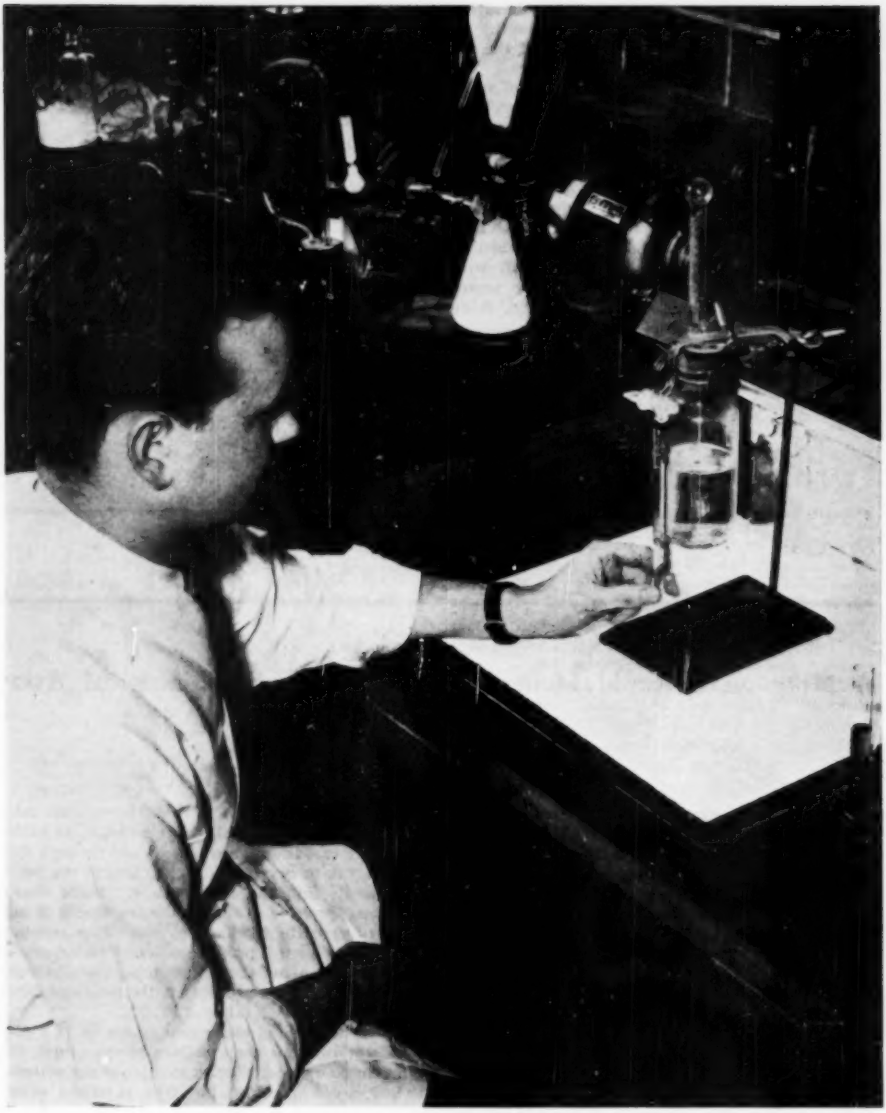

Fig. 1.—Apparatus used in rapid method for titrating microbiological assay culture tubes.

Book Reviews

Methods in Medical Research. Volume II. By JULIUS H. COMROE, JR., Editor-in-Chief. Year Book Publishers, Inc., Chicago, 1950. xvi + 361 pp. 14 x 22.5 cm. Illustrated. Price $6.50.

This is the second volume of a series intended to present complete, detailed methods useful in medical research. Each topic is presented by an expert in the field and represents, in many cases, the first time that these procedures have been written up in complete form.

The first section of this volume deals with Methods of Study of Bacterial Viruses. The author of the section describes fully the preparation of suitable media, isolation, techniques, assays of phage and host organisms, concentration and purification methods, and also several techniques used in studying the isolated virus, including growth rate determinations.

The second section, titled Pulmonary Function Tests, presents the techniques of preparing the patient as well as techniques used in making the tests. The apparatus used is described in detail with illustrations, and the mathematical handling of results, corrections of gas volumes, etc., are included. Measurement and analysis of expired gases, measurement of volume and flow rate, and measurement of gas tension in blood are also presented.

The Assay of Hormone Secretions is a particularly valuable contribution, this comprising the third section of the volume. The author divides the hormones into groups, anterior pituitary, ovarian, testicular and adrenal hormones, etc., and under each grouping gives both biological and chemical methods for assay of individual hormones.

The Pharmacopeia of the United States of America. Fourteenth Revision. Published by authority of The United States Pharmacopœial Convention, Inc. Mack Publishing Co., Easton, Pa., 1950. lv + 1067 pp. and supplement. 15.5 x 23.5 cm. Price $9; Outside U.S.A., $9.75.

The fourteenth revision of the Pharmacopeia of the United States is now available. In accordance with plans developed soon after the publication of U. S. P. XIII in 1946, the new edition was completed and a limited number of copies were available when the 1950 decennial U. S. P. Convention convened in May. Completion of the publication program was accomplished at that time so that responsibility for the U. S. P. XIV was not passed on to the new revision committee members who were elected during the recent U. S. P. Convention.

During a ten-year period the U. S. P. revision committee elected and organized in May, 1940, revised three editions of the U. S. P. This accomplishment of the 1940–1950 decade is equal in the number of revisions to that of a previous thirty-year period. In terms of the number of standards developed for new drugs, the revision activity of the 1940–1950 decade, however, is beyond comparison with any previous three revision periods of pharmacopeial history in the United States. A total of 204 new items has been added to U. S. P. XIV, an unprecedented number. This, however, is only a reflection of the rapid progress being made in the development of new potent and efficacious drugs. Not all of the new admissions to U. S. P. XIV represent what may be considered drugs of recent origin, but a substantial proportion represents developments of recent years.

Included in the list of new admissions are the antibiotics aureomycin hydrochloride, chloramphenicol (Chloromycetin), dihydrostreptomycin, streptomycin, tyrothricin, and dosage forms for each. Representative of the antihistaminic drugs diphenhydramine hydrochloride (Benadryl) and tripelennamine hydrochloride (Pyribenzamine) will be found among the new admissions. Other typical examples of new admissions are amphetamine sulfate (Benzedrine sulfate), calciferol, chloroguanide hydrochloride, chloroquine phosphate, dimercaprol (BAL), heparin sodium, meperidine hydrochloride (Demerol), naphazoline hydrochloride (Privine hydrochloride), pentylenetetrazol, (Metrozol), and vitamin B_{12}. In every instance a dosage form for each basic drug is included.

Of the monographs appearing in U. S. P. XIII, 119 were not included in U. S. P. XIV. Anyone not familiar with U. S. P. revision procedures might question the rationale of many of these deletions and also of many of the admissions. It is difficult to understand why it required nearly a century to establish the fact that aluminum acetate solution is a product worthy of recognition by the U. S. P., why mercury bichloride was dropped from U. S. P. XIII but reinstated in U. S. P. XIV, and why potassium nitrate was included in U. S. P. XII and XIV but not in U. S. P. XIII. On the whole, however, the admissions to U. S. P. XIV have been decided on an entirely rational basis.

The content of the U. S. P. is important even if it were intended to serve merely as a guide to the medical profession by including only drugs essential to the intelligent practice of medicine. If this had been the sole function through the years, however, a less pretentious book would have served that purpose. The main function of the modern Pharmacopeia is to serve as a book of standards for drugs. In this capacity it serves pharmacy, medicine, and the general public. The members of the pharmaceutical profession who contribute to the support of the pharmacopeial revision program either through direct participation in the work of revision or through purchase of the book should derive satisfaction from the results exemplified in the new fourteenth revision of the U. S. P.

BOOKS RECEIVED

A German-English Dictionary for Chemists. 3rd ed. By AUSTIN M. PATTERSON. John Wiley and Sons, Inc., New York, 1950. xviii + 541 pp. 13 x 17.5 cm. Price $5.

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Melting and Solidification of Fats. By ALTON E. BAILEY. Interscience Publishers, Inc., New York, 1950. xiv + 357 pp. Illustrated. 15.5 x 23.5 cm. Price $7.

Annual Review of Medicine. Vol. I. By WINDSOR C. CUTTING and HENRY W. NEWMAN. Annual Reviews, Inc., Stanford, Calif., 1950. vii + 484 pp. 15.5 x 23 cm. Price $6.

Prescription Writing and Materia Medica for Dentists. 3rd ed. By L. RICHARD CIPES. Dental Items of Interest Publishing Co., Inc., Brooklyn, 1950. xv + 530 pp. Illustrated. 16 x 24.3 cm. Price $8.50.

An Introduction to the Study of Experimental Medicine. By CLAUDE BERNARD. Henry Schuman, Inc., New York, 1949. xix + 226 pp. 15.5 x 23.5 cm. Price $3.

Proceedings of the First National Conference on Cardiovascular Diseases. Published by the American Heart Association. International Press, New York, 1950. xix + 259 pp. 15 x 23 cm. Price $1.75.

The Merck Manual of Diagnosis and Therapy. 8th ed. Merck and Co., Inc., Rahway, N. J., 1950. 1592 pp. 11 x 17.5 cm. Price $4.50; Thumb-Index Edition, $5.

1951 Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1951 Chilean Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine. Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1951 Award, nominations must be received on or before January 1, 1951.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The award consists of $1000 and a diploma setting forth the reasons for selection of the recipient. It may be presented annually at the annual meeting of the ASSOCIATION.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. In addition to the sum of the award, the recipient will receive an allowance of not more than $250 to defray his expenses in attending the meeting.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION'S Council. The present committee includes Justin L. Powers, chairman; Louis Gershenfeld, Charles O. Wilson, Harvey B. Haag, Heber W. Youngken, John C. Krantz, and Frank O. Taylor.

The award is now in its fourth year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist and the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University.

Dr. C. P. Leblond, Professor of Anatomy, McGill University, Montreal, Canada, will receive the third award and will give the 1951 Iodine Award lecture.

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